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THE ROLE OF AUTOPHAGY IN CARTILAGE PHYSIOLOGY AND METABOLISM: IMPLICATIONS FOR GROWTH AND AGEING

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Cover photo: LC3 puncta in hypertrophic chondrocytes of the mouse growth plate

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THE ROLE OF AUTOPHAGY IN CARTILAGE PHYSIOLOGY AND METABOLISM: IMPLICATIONS FOR GROWTH AND AGEING

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By

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Put your heart, mind and soul into even your smallest acts. This is the secret of success.

Swami Vivekananda

I would like to dedicate this thesis to

my beloved *Parents and Grandparents* and especially to grandmother *Ramanamma* who so very much desired for me to be successful in life.

ABSTRACT

Cartilage is the main constituent of the embryonic skeleton. At the ends of long bones cartilage forms a growth plate consisting of chondrocytes in distinct stages of differentiation and arranged into three zones. These chondrocytes mediate linear bone growth through synchronized proliferation, differentiation, and production of matrix. The cartilage lining the articulating surfaces of bones also contains chondrocytes arranged in different layers that secrete extracellular matrix and preserve cartilage integrity. Articular cartilage is relatively permanent, whereas the growth plate is transient. Although each of these cartilaginous structures has a unique structure and function, one fundamental similarity is that the chondrocytes in both are exposed to little blood and, thereby, low levels of oxygen and nutrients.

Autophagy is an intracellular pathway of lysosomal degradation that protects cells from both internal and external stressors and promotes cell viability when nutrition is limited. The protein kinase mTORC1 is a negative regulator of autophagy and its activity is, in turn, governed by various stimuli such as nutrition and growth factors, depletion of which inhibits mTORC1 and activates autophagy. Attenuated autophagy leads to various developmental and ageing-associated degenerative diseases.

Therefore, our primary hypothesis was that autophagy promotes chondrocyte survival, so, that inhibition of this process may impair the linear growth of bones and promote the development of age-related osteoarthritis. Our second hypothesis was that autophagy improves metabolic parameters during long-term intermittent caloric restriction.

First, we studied the role of autophagy in the chondrocytes of mouse metatarsal bones and in C5.18 cells by blocking this process with the lysosomal inhibitors bafilomycin A1 and chloroquine. We found that mTORC1 activity in chondrocytes was increased by blocking lysosomal V-ATPase enzymes. This effect is chondrocyte-specific and in contrast to well-accepted dogma. At the same time, inhibition of lysosomal activity stimulated the linear growth of mouse metatarsal bones by enhancing chondrocyte hypertrophy. Moreover, chondrocytes with impaired autophagy showed similar responses **(Paper I)**.

Subsequently, to investigate the effects of autophagy on linear bone growth **(Paper II)** and age-associated osteoarthritis **(Paper III)** directly we abrogated autophagy in chondrocytes by conditional deletion of the autophagy related Atg5 or Atg7 gene. We observed reduced axial and appendicular bone growth due to attenuated chondrocyte proliferation and elevated cell death in both cases. Moreover, chondrocyte viability in the human growth plate and mouse metatarsal bones was reduced by treatment with 3-methyladenine or bafilomycin A1, inhibitors of autophagy **(Paper II).** Fibrillations and proteoglycan loss in the articular cartilage of aged mice without a functional Atg5 gene was elevated indicating the development of osteoarthritis **(Paper III)**. These impaired bone growth and degenerative changes in articular cartilage are the consequences of enhanced apoptosis mediated by activation of caspases-3 and -9 **(Paper II and III)**. Furthermore, release of cytochrome C initiated the cleavage of caspases even in the absence of autophagy **(Paper II)**.

Finally, we examined the role of autophagy in metabolism during intermittent caloric restriction (according to a 5:2 diet) in obese individuals and with and without type II diabetes. We observed improvements in anthropometric and metabolic parameters in both our diabetic and non-diabetic subjects. Moreover, in diabetic subjects whose insulin sensitivity was improved by caloric restriction, autophagy also increased **(Paper IV)**.

In conclusion, the observations from our *in vitro* and *in vivo* studies confirm that autophagy is essential for the survival and homeostasis of chondrocytes in the growth plate and articular cartilage. At the same time, mTORC1 activation is chondrocyte-specific and independent of autophagy. In addition, autophagy improves metabolic parameters during intermittent caloric restriction in humans. Elucidating mTOR induced autophagy in greater detail will provide further insights in to disorders of linear growth, cartilage degeneration and metabolism, there by opening up novel approaches to treatment.

LIST OF SCIENTIFIC PAPERS

I. Phillip T Newton*, **KARUNA K VUPPALAPATI***, Thibault, Bouderlique and Andrei S Chagin. Pharmacological inhibition of lysosomes activates the mTORC1 signaling pathway in chondrocytes in an autophagy-independent manner. *Autophagy*, 2015, 11(9), 1594-1607.
*Equal contribution

II. **KARUNA K VUPPALAPATI**, Thibault Bouderlique, Phillip T Newton, Vitaliy O Kaminskyy, Henrik Wehtje, Claes Ohlsson, Boris Zhivotovsky, Andrei S Chagin. Targeted deletion of autophagy genes Atg5 or Atg7 in the chondrocytes promotes caspase-dependent cell death and leads to mild growth retardation. *Journal of Bone and Mineral Research*, 2015, 30(12), 2249-2261.

III. Thibault Bouderlique, **KARUNA K VUPPALAPATI**, Phillip T Newton, Lei Li, Björn Barenius, Andrei S Chagin. Targeted deletion of Atg5 in chondrocytes promotes age-related osteoarthritis. *Annals of the Rheumatic Diseases*, 2016, 75(3), 627-631.

IV. **KARUNA K VUPPALAPATI**, Neda Rajamand Ekberg, Michaela Sundquist, Ingeborg Eriksson, Jacob Grunler, Sergiu-Bogdan Catrina, and Kerstin Brismar. The metabolic effects of periodic fasting according the 5:2method in subjects with type 2 diabetes and subjects without diabetes but with adiposity. A sub-study of the effects on autophagy. *Manuscript in preparation.*

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LIST OF ABBREVIATIONS

3MA	3-methyladenine
ADAMTS-5	a disintegrin-like and metalloproteinase with thrombospondin type 1 motif, 5
AMPK	5'-adenosine monophosphate-activated protein kinase
ATG	AuTophaGy-related
ATG5cKO	ATG5 conditional knock-out
Baf	bafilomycin A1
BrdU	5-bromo-2 -deoxyuridine
CCN2	connective tissue growth factor-2
CQ	chloroquine
CR	caloric restriction
C5.18 cells	RCJ 3.1C5.18 rat mesenchymal cell line
COL2A1	collagen type II, α 1
ECM	extracellular matrix
FGF	fibroblast growth factor
FOXO	Forkhead box O
GAG	glycosaminoglycans
GC	glucocorticoids
GDF	growth differentiation factor
HIF-1	hypoxia-inducible factor-1
IGF-1	Insulin like growth gcator – 1
IGFBP-1	Insulin like growth factor binding protein-1
LAMP2	lysosomal- associated membrane protein 2
LC3	microtubule-associated protein 1A/1B-light chain 3
Mtor	Mechanistic (Mammalian) Target of Rapamycin
MMP	matrix metalloproteinase
OA	osteoarthritis
PPARγ	peroxisome-proliferator activated receptor γ

PTHrP	parathyroid hormone-related peptide
ROS	reactive oxygen species
Sumf1	sulfatase modifying factor 1
TSC	tuberous sclerosis complex
TUNEL	Terminal deoxynucleotidyl transferase-mediated deoxy-UTP Nick-End Labeling
UPR	unfolded protein response
V-ATPase	vacuolar-type H^+-ATPase

1 FOREWORD

This thesis focuses on the role of autophagy in connection with linear bone growth, osteoarthritis and metabolism under conditions of caloric restriction. The primary objective was to characterize the involvement of autophagy in chondrocyte survival and differentiation in the growth plate, as well as in the development and progression of osteoarthritis with ageing. In addition to its role in chondrogenesis, I attempted to elucidate the influence of caloric restriction on metabolism and autophagy. To achieve this, I employed an array of experimental models, including tissue-specific gene knockouts in mice, cell and bone cultures, modulation of autophagy by drugs, surgical intervention to induce arthritis, cultures of human growth plate and, finally, humans subjected to caloric restriction. Morphological, histological and molecular observations revealed the importance of autophagy in connection with cell viability, ageing-associated osteoarthritis, and metabolism.

2 INTRODUCTION

During times of stress, starvation, hypoxia, ageing and the like, self-regulatory autophagy provides a cell with the energy required to maintain its viability and homoeostasis. Recent advances in our understanding of the mechanism underlying this process have allowed exploration of its role in diverse pathophysiological conditions. The availability of nutrients and/or oxygen triggers signalling mechanisms that activate or inhibit autophagy, either directly or indirectly.

Our skeleton protects vital organs and provides support to and facilitates movement of the body. Its major components are chondrocytes and bone cells and its development depends on the synchronised kinetics of chondrocyte and osteoblast cells. Complex multifactorial processes govern skeletal growth, which begins as early as the third week of gestation and acquires definite form by week eight in humans. Genome–wide association studies have revealed that regulation of autophagy is involved in determining the adult stature of Han Chinese and whites in the United States (Pan et al. 2010), as well as bone mineral density (Zhang et al. 2010).

The cartilage in joints at the ends of bones buffers stress and supports body weight, and as a result of wear and tear, is more prone to injury and degeneration with time. In addition, metabolic diseases can cause an imbalance in bone remodelling, thereby promoting age-related disorders such as osteoarthritis. At present, no effective preventive or curative treatment for cartilage degeneration is available.

Intriguingly, recent insights into the importance of autophagy in chondrocytes have amplified the complexities of skeletal biology and cartilage homoeostasis during growth and ageing. Understanding and purposefully modulating autophagy might provide better ways to prevent or treat growth abnormalities and diseases of the cartilage. The current thesis focusses on linear bone growth, articular cartilage function and homoeostasis, autophagy and the mTOR pathway in connection with cartilage homeostasis in linear growth, ageing and osteoarthritis and metabolism under conditions of caloric restriction.

2.1 DEVELOPMENT OF THE SKELETON

The mammalian skeleton, which is mesodermal in origin, consists of cartilage and bone and is divided into the axial (vertebral column, skull, ribs, and sternum) and appendicular skeleton (limbs). The axial and appendicular skeleton develop from somites and lateral plate mesoderm, respectively, whereas cartilage and craniofacial bones originate from the cranial neural crest. The 206 bones that make up the human skeleton are formed through two well-organised processes referred to as intramembranous and endochondral ossification.

Intramembranous ossification involves direct transformation of mesenchymal cells into bone tissue which is how the bones of the skull, the clavicle and some facial bones are formed. Endochondral ossification, the formation of transient intermediate cartilage template that is

later replaced by bone is involved in the development of the long bones, vertebral column, ribs and sternum (Karsenty & Wagner 2017). The size of this template determines the size of the bone, which, in turn, depends on the number of precursor mesenchymal cells recruited to become chondrocytes (Hall & Miyake 1995).

2.1.1 Limb bud development

Limb bud formation and patterning begins during the fourth week of embryonic development in humans, and is completed by week eight (Gardner & O'Rahilly 1968). This development begins with mesenchymal condensation (Fig 1a and b) (Thorogood & Hinchliffe 1975), regulated by Hox genes, bone morphogenetic proteins (BMP), transforming growth factor-β and fibroblast growth factors (FGF) (Nelson et al. 1996; Long & Ornitz 2013). Following mesenchymal condensation, the cells differentiate into the chondrocyte lineage and begin to express SOX9 (Wright et al. 1995).

Prechondrogenic condensation and expression of SOX9 regulate chondrocyte differentiation, as well as the secretion of type II collagen, aggrecan and other matrix proteins (Hall & Miyake 1995; Kronenberg 2003). The cartilage template thus formed increases in size due to chondrocyte proliferation and differentiation coordinated by multiple signalling networks (Kronenberg 2003). Perichondrial cells migrate through invading blood vessels into the centre of this template, where they differentiate into osteoblasts. (Fig 1c and d) and as the bone and cartilage template lengthen, a secondary ossification centre appears (Fig 1e).

The growth plate cartilage, a highly-specialised tissue with a significant role in shaping the skeleton develops between the two centres of ossification. The successful development of cartilage requires maintaining proper skeletal proportions in the face of many challenges and the chondrocytes continue to proliferate and differentiate to increase bone length until the growth plate fuses (Kronenberg 2003).

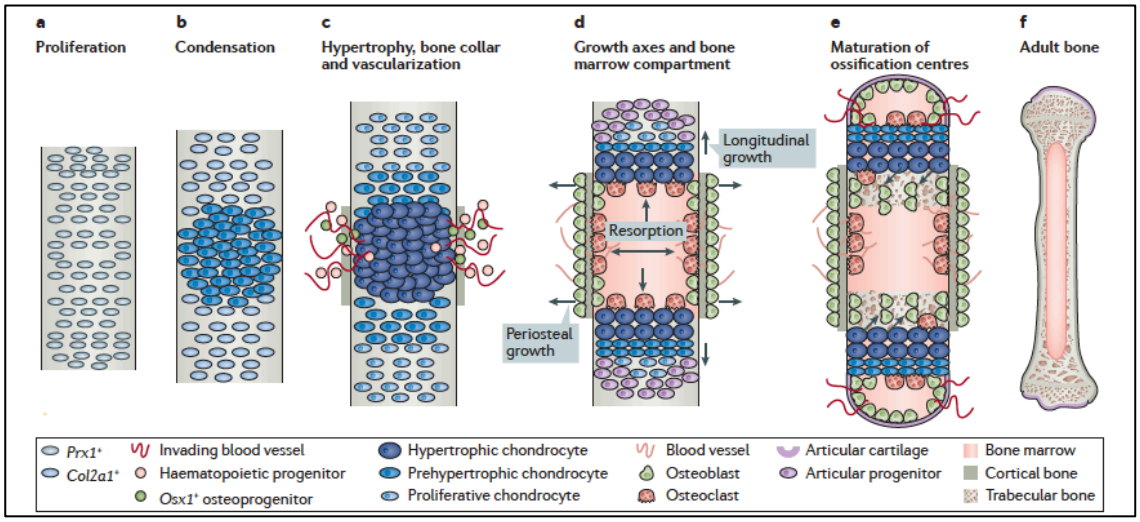

Figure 1. **Developmental skeletogenesis**. Longitudinal views depicting key steps of endochondral bone formation in mouse limbs. **a** | *Prx1*$^{+}$ progenitors from lateral plate mesoderm proliferate to populate the emerging limb bud. **b** | Cells nearest the centre undergo mesenchymal condensation, express *Col2a1* as they enter a chondrogenic differentiation program, and deposit a cartilage template. **c** | to **d** | Differentiating cells upregulate *Col10a1* as they become hypertrophic, which triggers local formation of a bone collar and vascularization of the cartilage template. Invading blood vessels deliver an influx of haematopoietic cells that give rise to osteoclasts which excavate the cartilage template, and *Osx1*$^{+}$ osteoblast progenitors and other blood cell types that populate the newly formed marrow cavity. **d** | A longitudinal growth axis is established when vascularization and osteoclast-mediated resorption bisect the presumptive skeletal element, producing two growth plates with opposing directionality. A perpendicular growth axis is driven by periosteal osteoblasts and allows the bone to grow in width. **e** | Within the remodelled cartilage template, bone-forming osteoblasts are derived from *Osx1*$^{+}$ cells arriving with the invading vasculature, as well as hypertrophic *Col10a1*$^{+}$ chondroctyes that transdifferentiate as they exit the growth plate into the marrow cavity. As bones grow in length and width, a second wave of vascularization forms the secondary ossification centres. **f** | Mature endochondral bone.
Illustration reprinted by permission from Macmillan Publishers Ltd: BMP signalling in skeletal development, disease and repair by Gamer, L. W. & Rosen, V. et al. Nat. Rev. Endocrinol. doi.10.1038/nrendo.2016.12, copyright (2017).

2.1.1.1 Longitudinal bone growth

In humans, longitudinal bone growth at the epiphyseal growth plate is rapid during the early years of life, decreases gradually thereafter to reach a plateau after the pubertal growth spurt, and ceases with the fusion of the growth plate. With the exception of pronounced growth spurt that occurs during puberty in humans, this same pattern is observed in other mammals (Tanner & Davies 1985).

2.1.1.2 Structure and function of the growth plate

The growth plate, also called the physis or epiphysis, is a transient layer of hyaline cartilaginous tissue composed exclusively of chondrocytes derived from multipotent skeletal progenitors. Cascading of these progenitor cells through various lineage commitments and differentiation steps leads finally to the formation of a highly organised growth plate with three distinct zones (Fig 2) (Liu et al. 2017; Sun & Beier 2014).

Resting zone: The region near the epiphysis is referred to as the resting zone and contains round undifferentiated chondrocytes believed to serve as stem-like cells that generate proliferating chondrocytes. The stem-like and proliferative properties of these cells decline with age and finally disappear following puberty (Abad et al. 2002).

Proliferative zone: Chondrocytes in the resting zone are recruited into the proliferative zone, where they form several proliferating columns aligned parallel to the long axis of the bone (Abad et al. 2002; Kronenberg 2003). Chondrocyte proliferation accounts for approximately 10% of total growth (Wilsman et al. 1996) and these cells secrete type II collagen (Kronenberg 2003).

Hypertrophic zone: The chondrocytes located farthest from the epiphysis stop proliferating, and enlarge almost 15-fold in size (Bush et al., 2010). This hypertrophy involves three distinct phases: During phases I and III dry mass and volume increase concomitantly while in phase II only the volume increases. The rate of change during phase III is regulated by Insulin-like Growth Factor 1(IGF-1) and characterises the bone as either fast- or slow-growing (Cooper et al. 2013).

These hypertrophic chondrocytes secrete enormous quantities of extracellular matrix, including type X collagen, factors such as Ihh that promote the differentiation of chondrocytes and vascular endothelial growth factor (VEGF), which attracts blood vessels. Finally, these chondrocytes die through some as-yet-unidentified mechanism and are replaced by osseous tissue. This zone is responsible for almost 50-60% of bone growth with a remarkable turnover of these hypertrophic chondrocytes within a span of only 24 hours (Wilsman et al. 1996; Bush et al. 2010)

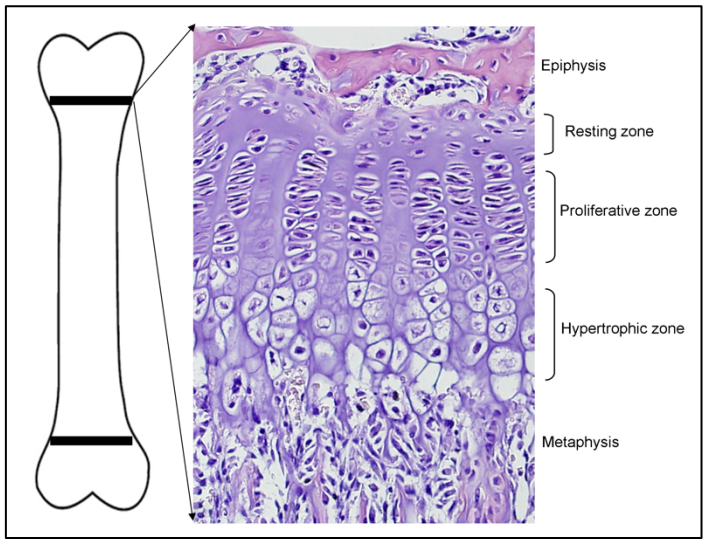

Figure 2. The structure of the growth plate. Hematoxylin and Eosin staining of the mouse growth plate shows the resting, proliferative and hypertrophic zones.

Thus, in summary, growth plate cartilage consists of transient highly active chondrocytes that undergo proliferation, hypertrophy, maturation, and cell death, being eventually replaced by bone tissue.

Regulation of growth plate: Several autocrine and paracrine signalling pathways regulate growth plate kinetics. The primary endocrine regulation involves the GH/IGF-1 axis as well as thyroid hormone, androgens, oestrogens and glucocorticoids (Nilsson et al. 2005; van der Eerden et al. 2003).

Local regulation involves paracrine and autocrine signalling by Indian hedgehog (Ihh) and parathyroid hormone-related peptide (PTHrP), which act through a positive-negative feedback loop that governs chondrocyte exit from the proliferative and entry into the hypertrophic zone (Kronenberg 2003). Other factors include FGFs, BMPs and Wnts (van der Eerden et al. 2003; Chagin & Sävendahl 2009; Nilsson et al. 2005). In addition to these systemic and local regulations, the tissue microenvironment plays a vital role in coordinating cellular kinetics, either alone or in conjunction with hormonal pathways.

2.1.1.3 The fate of hypertrophic chondrocytes

The removal of terminally differentiated chondrocytes from the growth plate has been proposed to involve transdifferentiation, apoptosis and autophagy.

Transdifferentiation: Initial observations on the avian growth plate indicated that chondrocytes transdifferentiate into osteoblasts (Roach 1992) and in reporter mouse models this is the fate of more than half of the chondrocytes. This transformation begins from the cartilage template in the embryonic period, even before the establishment of growth plates (X. Zhou et al., 2014, Liu Yang et al., 2014). Recently, lineage tracing in Col10A1-cre; RYFP and Col10A1-Cre; GFP (Collagen X, specific marker of hypertrophic chondrocytes) transgenic mice revealed the presence of chondrocyte-derived YFP-positive cells resembling osteoblasts at the chondro-osseous junction and in the endosteum and cortical bone matrix, indicating that chondrocytes transdifferentiates into osteoblasts and osteocytes (G. Yang et al. 2014).

Apoptosis: Farnum and Wilsman (1989) observed cellular condensation of hypertrophic chondrocytes in the mammalian growth plate and proposed that hypertrophic chondrocytes are eliminated from the growth plate during endochondral ossification by apoptosis (Farnum & Wilsman 1989). Apoptosis also occurs among hypertrophic chondrocytes in the chick (Hatori et al., 1995) and rabbit growth plates (Aizawa et al., 1997). The frequency of apoptotic cells increases with age and, moreover, terminal hypertrophic chondrocytes express caspase -3 and -6, indicative of apoptosis (Chrysis et al., 2002). The occurrence of apoptosis in the growth plate has also been confirmed by the finding that chondrocyte-specific deletion of the pro-apoptotic Bcl-x gene elevates the cellular levels of caspase -7 and shortens the hypertrophic zone (Oshima et al. 2008).

Autophagy: Emons and colleagues (2009) found that chondrocytes in the fusing human growth plate do not exhibit features of either apoptosis or autophagy (Emons et al. 2009). Later, hypertrophic chondrocytes in Meckel's cartilage were shown to express LC3 and Beclin1 markers of autophagy prior to their removal by apoptosis (R.-T. Yang et al. 2012).

2.1.2 **Joint development**

Joint development begins with the appearance of a mass of blastedermal cells near the space of future joint, with subsequent development of an interzone within this mass (Holder 1977) the first sign of joint formation (Holder 1977; Koyama et al. 2008). The joint later forms

through differentiation of chondrocytes in the interzone (Hartmann & Tabin, 2001), which consists of three layers of cells: two outer facing the epiphyseal end of long bones and a dense intermediate layer. Any disruption of this zone leads to malformation of joints or the fusion of adjacent bones (Holder 1977). After cell differentiation, joint cavitation facilitated by hyaluronan develops in a proximal to distal direction (C W Archer et al. 1994).

Growth plate chondrocytes and articular cartilage cells have different origins and only the latter provide support throughout life. Articular chondrocytes are derivative from cells that express Gdf5 (Growth/Differentiation factor 5), whereas epiphyseal chondrocytes are Gdf5-negative (Settle Jr. et al. 2003; Merino et al. 1999; Koyama et al. 2008). Other important factors involved in joint formation include Wnt9a, which signals the chondrocytes to establish the joint (Hartmann & Tabin, 2001) and noggin which regulates Gdf5 expression (Brunet et al. 1998).

2.1.2.1 The structure, histology and function of articular cartilage

Articular cartilage is located in diarthrodial joints, where it transmits the mechanical load and provides a smooth and lubricating surface for the movement of adjacent articulating bones. This is hyaline cartilage, devoid of vessels and nerves, 2 - 4 mm thickness (Hunziker et al. 2002; Sophia Fox et al. 2009) and consisting of chondrocytes and an extracellular matrix composed of water, collagen and proteoglycans. It is divided into superficial, middle and deep zones (Fig 3).

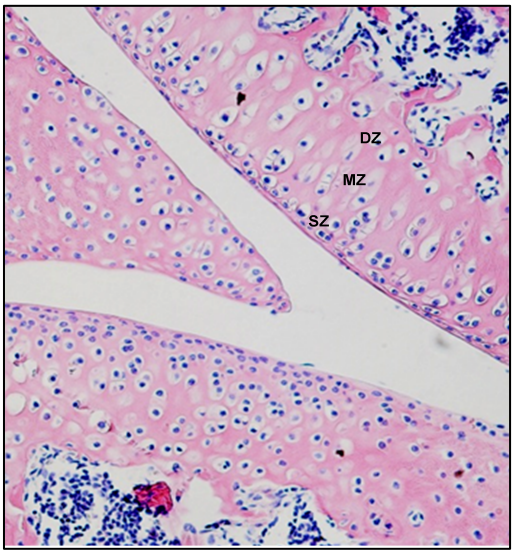

Figure 3. Structure of the murine articular cartilage. Hematoxylin and Eosin staining shows the SZ – superficial zone, MZ –middle zone and DZ –deep zone.

The superficial zone consists of small densely packed flat chondrocytes and type II and IX collagen all arranged parallel to the articular surface (Hunziker et al. 2002). As progenitors for the other types of chondrocytes, these cells participate in joint development (Li et al. 2016). This zone is in closest contact with the synovial fluid and the chondrocytes in the superficial layer secrete lubricin into this fluid. The primary function of the superficial zone is to protect the deep layers from shear stress (Ikegawa et al. 2000).

The middle zone consists of sparsely packed spherical chondrocytes, proteoglycans and dense collagen fibrils aligned in a direction oblique to the articular surface. Finally, the deep layer consists of chondrocytes in columns, collagen fibrils whose largest diameter is arranged perpendicular to the articular surface, and large amounts of proteoglycan to resist mechanical forces (Broom & Poole 1982; Sophia Fox et al. 2009).

The most abundant form of collagen and proteoglycan in articular cartilage is type II, and aggrecan respectively. The chondrocytes there occupy only 2% of total volume, but are important regulators of cartilage homoeostasis, balancing synthesis, repair and degradation of the ECM (Archer & Francis-West 2003). Despite their considerable metabolic activity, these chondrocytes have very limited capacity to replicate and do not establish cell-to-cell interactions (Sophia Fox et al. 2009).

Several growth factors such as IGF-1, transforming growth factor and interleukin 1 are involved in proteoglycan metabolism, while matrix metalloproteinases (MMP) degrade cartilage. Physical factors like joint loading and unloading are important in maintaining healthy cartilage, with inactivity, ageing and/or joint injury leading to an imbalance in the homeostasis of the cartilage and extracellular matrix. With advancing age the number of chondrocytes decreases in superficial zone and increases in the deep zone and, moreover, the water content of articular cartilage is reduced resulting in joint stiffness (Sophia Fox et al. 2009; Martin & Buckwalter 2001).

2.2 OSTEOARTHRITIS

2.2.1 Incidence, risk factors and histological features

Osteoarthritis (OA), the most common degenerative disease affecting joints develops most often in knee and hip (Cross et al. 2014). Worldwide almost 50% of individuals older than 65 are afflicted by this disease and the incidence continuous to increase, making OA the 11th most serious cause of general debility globally today (Cross et al. 2014). This complex musculoskeletal disease is characterised by the degradation of the extracellular matrix, a reduction in the number of chondrocyte and formation of clusters of these cells, irregularities in the joint surface, synovial inflammation, osteophyte formation, fibrosis of the joint capsule and remodelling of subchondral bone (Lotz & Carames 2011).

Many factors, both systemic and local, involved in the development and progression of this disease includes ageing, obesity, gender, genetic factors and injury to menisci or ligaments (Martel-Pelletier et al. 2012). Manifestation occurs only rarely at a young age, when cartilage degeneration following joint injury can be controlled more effectively than at a more advanced age (Gelber et al. 2000; Roos 2005). Moreover, genome-wide association studies have revealed that genes located on chromosome 7q22 play a role in the development of the OA of the knee (Evangelou et al. 2011).

Several pathophysiological mechanisms underlie the destruction of cartilage. The early microscopic changes detected in OA cartilage include fibrillations in the superficial zone and loss of proteoglycans. As the disease advances, clefts and fissures appear deep within the cartilage. All of these changes result in matrix degradation, chondrocyte differentiation and leakage of inflammatory mediators into the joint space (Lotz & Carames 2011).

2.2.1 Cellular changes during ageing and osteoarthritis

The superficial zone contains mesenchymal stem cells that express high-mobility group box protein 2 (HMGB2), as well as proteoglycan 4 or lubricin. HMGB2 promotes cell survival, and lubricin is an essential constituent of synovial fluid that protects the cartilage from external compressive forces. Severe mechanical damage to the superficial zone results in the production of matrix-degrading enzymes. In addition, defects in lubricin production lead to joint pathologies such as OA, and ageing attenuates the expression of both HMGB2 and proteoglycan 4 in the superficial zone. Moreover, superficial zone chondrocytes express matrix metalloproteinases and inflammatory cytokines that contribute to the pathogenesis of OA (Tetlow et al. 2001).

The chondrocytes in articular cartilage are post-mitotic and undergo very little turnover, a state referred to as "chondrocyte phenotype senescence". Due to this reduced capacity for replication, damaged organelles and/or long-lived or misfolded proteins may accumulate with time. Furthermore, mitochondrial ageing leads to oxidative damage from reactive oxygen species (ROS), one of the factors proposed to cause cellular senescence and referred to as "stress-induced senescence". The decrease in chondrocyte number in OA in connection with advancing age and OA is attributed to an imbalance between the production of reactive oxygen species and antioxidant defence mechanisms (Jallali et al. 2005). These age-related alterations in cartilage can be delayed by active autophagy.

2.3 AUTOPHAGY AND mTOR

2.3.1 Autophagy

The prestigious Nobel Prize in Physiology or Medicine 2016 has been awarded to Professor Yoshinori Ohsumi for his “discovery of mechanisms for autophagy” (nobelprize.org).

Although the term autophagy was first coined by Christian De Duve after his discovery of lysosomes in 1960 (Deter & de Duve 1967), the physiological importance of this phenomenon was poorly understood for a long time and only during the 1990’s were proteins that regulate autophagy first discovered.

As a complement to protein degradation via the ubiquitin-proteasome pathway, which is highly selective and degrades short-lived proteins specifically, autophagy degrades both short- and long-lived proteins (Mizushima & Komatsu 2011). The three different forms of autophagy in mammalian cells – macroautophagy, chaperone-mediated autophagy and microautophagy (Fig 4) -- differ with respect to cargo degradation (Glick et al. 2010).

As the first step in autophagy, a highly-conserved process involving lysosomal degradation, cellular components such as, damaged organelles and long-lived proteins are sequestered into a double-membrane vesicle called the autophagosome. Later the mature autophagosome fuses with lysosomes and releases its contents along with its inner membrane into the lysosomal lumen. Thereafter, lysosomal enzymes digest the contents and their components are released

back into the cytoplasm (Fig 4A) for re-cycling designed to maintain cell viability under stressful conditions. This entire process is usually referred as macroautophagy (hereafter, autophagy) (Levine & Daniel J Klionsky 2004; Mizushima & Komatsu 2011; Glick et al. 2010).

The degradation of cytoplasmic proteins containing a specific pentapeptide sequence (KFERQ) that is recognised by chaperone protein HSC70 is defined as chaperone-mediated autophagy (Fig 4B) (Orenstein & Cuervo 2010). Finally, the direct uptake of cytoplasmic constituents into lysosomes by invagination of the lysosomal membrane is called microautophagy (Fig 4C) (Sahu et al. 2011).

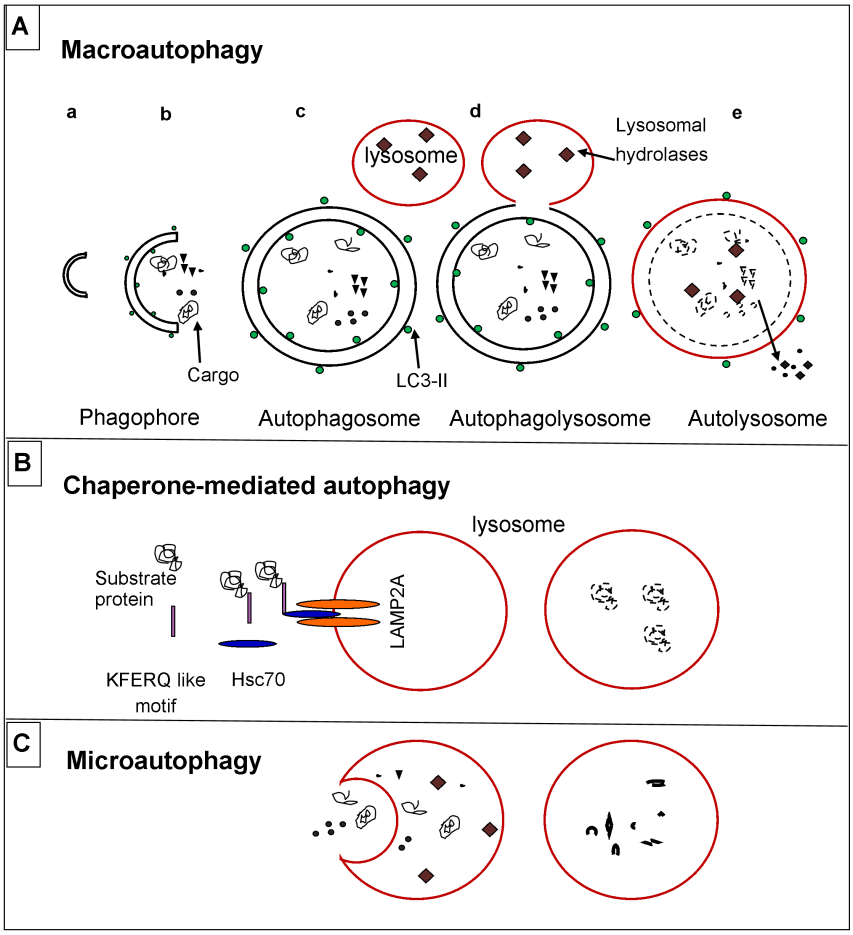

Figure 4. The different forms of autophagy. (A) The sequence of events in macroautophagy: formation of the phagophore, sequestration of the cargo, autophagosome formation, the fusion of the autophagosome with the lysosome, degradation of cargo by lysosomal enzymes and release of basic constituents back into the cytoplasm. **(B)** The sequence of events in chaperone-mediated autophagy: identification of proteins containing an KFERQ motif by chaperone Hsc70, binding of the protein complex to LAMP-2A and translocation into the lysosome followed by degradation. **(C)** The sequence of events in the microautophagy: direct uptake of cytoplasmic constituents by invagination of the lysosomal membrane and their subsequent degradation.

Autophagy can be both selective or non-selective. Non-selective autophagy recycles bulk cytoplasm, whereas selective autophagy involves sequestration of specific components such as mitochondria, the endoplasmic reticulum, peroxisomes, microbes, etc. (Glick et al. 2010).

2.3.1.1 The Autophagic machinery

Autophagy is regulated primarily by the products of autophagy-related (ATG) genes, about 40 genes of which have been identified in *Saccharomyces cerevisiae* and *Pichia pastoris* to date (Feng et al. 2015; He & Klionsky 2009; Glick et al. 2010). This process involves five key steps –" i) Formation of the phagophore (nucleation), ii) Atg5-Atg12-Atg16L conjugation (elongation), iii) processing of LC3 and its recruitment to the autophagosome membrane (autophagosome formation), iv) uptake of cargo and v) fusion of the autophagosome with a lysosome" (Fig 5) (Glick et al. 2010).

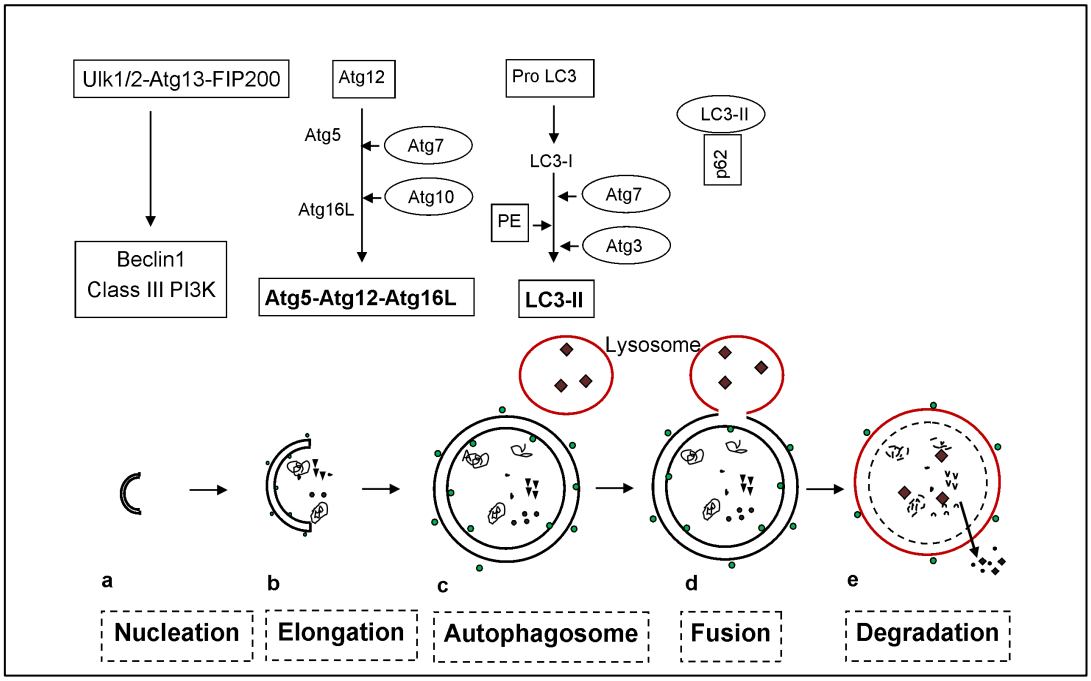

Figure 5. The autophagy machinery. (a) The Ulk1/2 and Beclin1 complexes encoded by autophagy-related genes initiate nucleation (b) Formation of the Atg5–Atg12-Atg16L complex leads to extension of the phagophore and (c) formation of the autophagosome (b, c). Soluble LC3 I is converted to membrane-bound LC3 II, which binds to adaptor protein p62 (d, e). Fusion of the autophagosome with a lysosome and cargo degradation by lysosomal enzymes.

Beclin1, which is homologous to yeast Atg6, regulates the initiation of autophagy and phagophore formation through Class III PI3K (Fig 5a). Phagophore formation begins with formation of the Atg1-Atg13-Ag17 (Ulk1/2-Atg13-FIP200 in mammals) complex, and extension of the phagophore is associated with the formation of the Atg5–Atg12-Atg16L multimeric complex (Kuma et al. 2002). This latter complex is formed upon activation of Atg12 by Atg7 (Tanida et al. 1999), which acts like the ubiquitin-activating enzyme E1 (Hanada et al. 2007). Induction of autophagy involves the cleavage and activation of soluble cytosolic LC3-I by Atg4, following which activated LC3-I is conjugated with phosphatidylethanolamine to yield LC3-II, which is then incorporated into the autophagosomal membrane. A second ubiquitin-like system involving Atg3 plays a role in

activating the cleavage site of LC3 (Fig 5) (Ichimura et al. 2000). During active autophagy, both the synthesis and processing of LC3 are elevated, making LC3 a marker of autophagy.

Fusion of the autophagosome with the lysosome is mediated by the small G protein called Rab7 (Jäger et al. 2004) and LAMP1 and LAMP2 play significant roles during the final stages of autolysosome maturation. The lysosomal enzymes cathepsin B and D digest the cargo delivered (He & Klionsky 2009; Glick et al. 2010). The central regulatory kinase is mTOR, which inhibits initiation of autophagy by phosphorylating Ulk1/2 (Atg1 in yeast).

Several investigations have shown that autophagy can occur independently of conventional ATG genes, a process referred to as non-canonical autophagy (Codogno et al. 2012). Ulk1/2-independent autophagy in MEFs has been reported during glucose deprivation of ULK1/2 double-knockout mice (Cheong et al. 2011). Moreover, Atg5/Atg7-independent autophagy is observed during erythrocyte maturation (Nishida et al. 2009). Although the mechanism by which, the autophagosome is generated is different, the terminal lysosomal degradation resembles the classical pathway (Codogno et al. 2012).

2.3.1.2 Regulation of autophagy

Autophagy can be activated or inhibited by the various intra- and extracellular signals discussed below.

Nutrients: The most well-studied of these is starvation, which activates several pathways and transcription factors that modulate autophagy. Nutrients, and in particular amino acids, inhibit autophagy by activating the mTORC1 pathway (Long et al. 2005). In contrast, rapamycin activates autophagy under nutrient-rich conditions by inhibiting mTORC1 (Jung et al. 2009). Glucose activates the PKA pathway and inhibits autophagy via AMPK, which in turn regulates the mTOR pathway (Budovskaya et al. 2004). During nutrient deprivation, transcription factors such as Forkhead box O stimulate the expression of autophagy-related genes (Sengupta et al. 2009).

Growth factors: Growth factors phosphorylate and activate PKB/Akt, which in turn stimulates mTORC1 by inhibiting formation of the TSC1/TSC2 complex by phosphorylating TSC2 (Inoki et al. 2002), thereby inhibiting autophagy (Fig 7) (He & Klionsky 2009).

Energy: The activity of AMPK, the primary sensor of the level of cellular energy, rises as the AMP/ATP ratio increases (Hardie 2007). AMPK activates autophagy by inhibiting mTOR via TSC1/2 (Meijer & Codogno 2007; Inoki et al. 2003).

Hypoxia: Hypoxia, either physiological or pathological, is also involved in the regulation of autophagy. The hypoxia-inducible factor is the major transcription factor that regulates the expression of various genes in a manner designed to promote survival in the presence of low levels of oxygen. Hypoxia induces autophagy by activating BNIP3 and BNIP3L (Bellot et al. 2009). mTORC1 stimulates HIF1α transcription through the 4E-BP1-eIF4

axis (Düvel et al. 2010). Moreover, hypoxia inhibits mTOR activity by increasing thenobel prize.org

expression of REDD1, which in turn acts through the TSC1/2 pathway (Brugarolas et al. 2004).

In addition to the factors mentioned above, others such as ER stress, reactive oxygen species (ROS), infections and epigenetic changes can modulate autophagy (He & Klionsky 2009).

The physiological functions of autophagy: Autophagy provides components, for the renewal of cellular constituents, as well as for energy production. This process regulates cell size, differentiation and overall cellular homoeostasis during growth, as well as during ageing. In addition, it plays a significant role in eliminating intracellular microorganisms and preventing tumour formation. Overall, autophagy regulates the quality of the intracellular environment (Boya, Reggiori, & Codogno, 2013; Levine & Klionsky, 2004).

2.3.2 **Mechanistic target of rapamycin (mTOR)**

Mechanistic (Mammalian) target of rapamycin is an evolutionarily conserved, nutrient-sensing, serine-threonine kinase that maintains the balance between anabolism and catabolism thereby regulating cellular homoeostasis. mTORC1 is composed of mTOR, mLST8 and raptor. And mTORC2 is made up of mTOR, mLST8 and rictor (Guertin & Sabatini 2007).

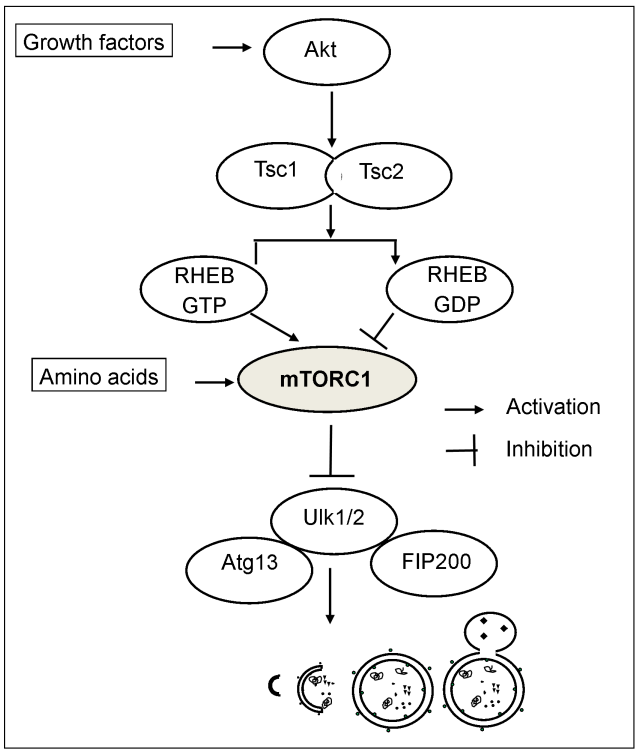

Figure 6. Regulation of mTORC1. Activation of Akt by growth factors inhibits TSC1/2, which in turn inhibits mTORC1 by acting as the GTPase-activating enzyme Rheb. Amino acids act directly on mTORC1. Activated mTORC1 prevents formation of the Ulk1/2 complex, thereby inhibiting autophagy.

2.3.2.1 Regulatory mechanisms involving mTORC1:

Regulation of autophagy: Under nutrient-rich conditions mTOR inhibits autophagy by phosphorylating Ulk1/2 and Atg13. In contrast, nutrient deprivation or inhibition of TOR induces autophagy by enhancing Ulk1 kinase activity, thereby leading to phosphorylation of FIP200 (focal adhesion kinase family interacting protein of 200 kDa) and Atg13 (Fig 6). (Jung et al. 2009)

Regulation by growth factors and nutrients: In the presence of growth factors and nutrients mTORC1 is activated via various mechanisms. Growth factors activate mTOR through the PI3K/Akt pathway, leading to the phosphorylation and thereby inhibition of the tuberous sclerosis complex (TSC1/2) (Inoki et al. 2002). This complex is a GTPase-activating enzyme that inhibits mTORC1 by suppressing Rheb (Ras homologue enriched in brain). Rheb in its active GTP-bound state is required for activation of mTOR by all upstream signals (Fig 6) (Laplante & Sabatini 2009; Roccio et al. 2005).

Nutrient-rich conditions, and especially amino acids facilitate the translocation of mTORC1 to the lysosomal surface, in a manner independent of growth factors, TSC or Rheb activity. Rag GTPases located on the lysosomal membrane are involved primarily in the activation of mTORC1 (Sancak et al. 2010). The interaction between Rags and mTOR and subsequent translocation in the presence of amino acids is regulated by vacuolar H^+-adenosine triphosphatase ATPase (V-ATPase) (Efeyan et al. 2012).

2.3.2.2 Autophagy and mTORC1 in relationship to lysosomal function

Autophagy requires functional lysosomes and lysosomal function is also dependent on ATG genes and autophagosome-lysosome fusion (Zhou et al. 2013). Moreover, lysosomal synthesis depends on the transcription factor EB (TFEB), the activity of which is controlled by mTORC1 (Sardiello, et al. 2009). In the other direction, mTORC1 activity is controlled by lysosomal position and amino acid sensing by lysosomes.

Interference with mTORC1 function or starvation leads to nuclear translocation and increased transcriptional activity of TFEB, thereby promoting autophagy (Peña-Llopis et al. 2011; Zhou et al. 2013). TFEB regulates V-ATPases on the lysosomal membrane in a manner dependent on the availability of nutrients (Hinton et al. 2009). Disruption of lysosomal function with drugs such as Baf, CQ and concanamycin prevents V-ATPases and Rags from performing their actions, thus inhibiting mTORC1 activation and its translocation to the lysosomal membrane (Li et al. 2013). Our recent findings indicate that, the universally accepted phenomenon of mTORC1 inhibition by lysosomal blockage is cell specific, i.e., in terminally differentiated chondrocytes and osteoclasts, inhibition of lysosomal V-ATPase activates mTORC1 (Newton et al. 2015; Hu et al. 2016).

Physiological functions: mTORC1 regulates cell growth and differentiation by inducing anabolic processes such as protein and lipid synthesis. Activated mTORC1 regulates protein synthesis by phosphorylating its downstream targets p70S6Kinase and the eukaryotic translational initiating factor 4E binding protein (Richter & Sonenberg 2005).

2.3.3 The function and regulatory mechanism of mTORC2

mTORC2 regulates cell polarity, organisation of the cytoskeleton and overall cell survival (Jacinto et al. 2004) by phosphorylating several downstream targets, including Akt, protein kinase c alpha and serum and glucocorticoid-induced protein kinase 1 (Oh & Jacinto 2011). Signalling molecules such as IGF-1 (Yin et al. 2016) and wnt (Esen et al. 2013) and local

factors such as mechanical stress (Rangaswami et al. 2012; Zeng et al. 2015) also act through the mTORC2 pathway. mTORC2 is generally insensitive to amino acids and rapamycin, although prolonged exposure of certain types of cells to rapamycin does inhibit its action (Sarbassov et al. 2006).

Disruption of the mTOR pathway has been implicated in the development of several diseases, including diabetes, cancer, neurodegenerative diseases, etc. (Laplante & Sabatini 2013).

2.4 MODULATORS OF AUTOPHAGY AND mTOR

2.4.1 Activators of autophagy

Starvation and *hypoxia* both activate autophagy, as discussed in detail in section 2.3.1.2. above.

Endoplasmic reticulum stress: Endoplasmic reticulum stress resulting from the accumulation of misfolded proteins is a highly potent activator of autophagy, acting through inhibition of the Akt/TSC/mTOR pathway (Lotz & Carames 2011; Ogata et al. 2006; Bachar-Wikstrom et al. 2013).

Rapamycin, widely used as an immunosuppressant in connection to renal transplantations, inhibits mTORC1 specifically (Fig 7) by forming a complex with the FK506 binding protein 12 (FKBP12) and inhibiting its activity (Guertin & Sabatini 2007). Temsirolimus and Everolimus, derivatives of Rapamycin, are currently being tested in clinical trials (Ballou & Lin 2008).

Trehalose, a disaccharide that protects against cellular stress, activates autophagy in an mTOR-independent manner (Sarkar et al. 2007, Mizushima et al. 2010).

Lithium chloride, used to treat bipolar disorders, also activates autophagy in an mTOR-independent manner, in this case by inhibiting inositol monophosphatase (Sarkar et al. 2005; Sarkar & Rubinsztein 2008; Yang et al. 2013).

2.4.2 Inhibitors of autophagy

3-methyladenine (3MA), a class III inhibitor of PI3K, interferes with formation of the autophagosome (Fig 7). Although widely considered to be an inhibitor of autophagy, the presence of 3MA under nutrient-rich conditions activates autophagic flux, but inhibits starvation-induced autophagy (Wu et al. 2010; Klionsky et al. 2016).

Bafilomycin A1(Baf), a macrolide antibiotic isolated from *Streptomyces* species, is a highly specific and potent inhibitor of vacuolar type H+-ATPase (V-ATPase)(Crider et al. 1994) thereby blocking the acidification of lysosomes or other organelles. Disruption of the acidification of lysosomes inhibits their fusion with the autophagosome (Fig 7) (Mauvezin & Neufeld 2015; Klionsky et al. 2016; Mizushima et al. 2010). Although widely used in research, treatment of humans with Baf is limited by its extensive toxicity and narrow therapeutic window (Keeling et al. 1997).

Chloroquine, a traditional anti-malarial drug, inhibits autophagy by inhibiting lysosomal acid hydrolases (Fig 7), thereby rendering the lysosomal pH more alkaline (Geng et al. 2010; Klionsky et al. 2016).

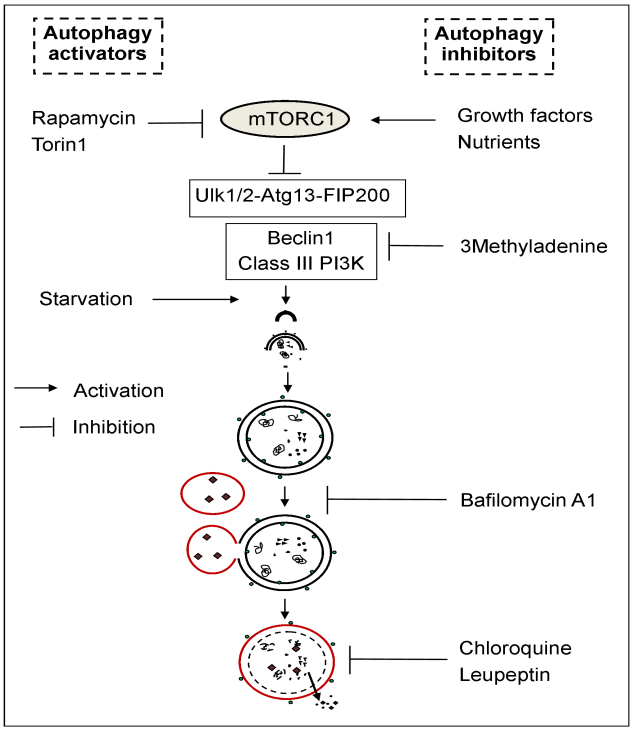

Figure 7. Activators and inhibitors of autophagy. Growth factors and nutrients act through mTORC1 and inhibit autophagy, mTOR inhibitor rapamycin activates autophagy, whereas 3Methyladenine inhibits autophagy by blocking initiation and baf, CQ and leupeptin inhibit fusion of autophagosome.

Leupeptin inhibits lysosomal proteases, thereby inhibiting degradation of the cytoplasmic contents of autophagosomes within lysosomes (Fig 7) (Haspel et al. 2011).

The compounds mentioned above are widely used in experiments designed to understand the mechanism(s) underlying autophagy and interactions of mTOR with various signalling pathways.

2.5 CELL DEATH

Cell death plays a major role in maintaining homoeostasis, both within and outside tissue, *e.g.,* during development, in connection with immune responses and pathophysiological conditions. Previously, cell death was defined as either apoptosis and necrosis (Kerr et al. 1972), the former being defined as genetically programmed cell death and the latter as passive cell death. With the discovery of autophagy, cell death is now classified into three different types on the basis of morphological and histological features (Golstein & Kroemer 2007).

2.5.1 Types of cell death

Apoptosis or type I programmed cell death, a physiological process during development can be triggered by both physiological and pathological events. This process is divided into extrinsic (or death receptor-mediated) and intrinsic (or mitochondrial-mediated) apoptosis

(Elmore 2007), both of which are characterized by chromatin and cytoplasmic condensation, pyknotic nuclei, DNA fragmentation and cell shrinkage (Kerr et al. 1972).

Autophagy or Type II programmed cell death, which also occurs under both physiological and pathological conditions, is characterised by the presence of cytoplasmic vacuoles (Golstein & Kroemer 2007). In some tissues, autophagy and apoptosis coexist, or apoptosis may precede autophagy (Bursch et al. 2000). Recently, the Nomenclature on Cell Death specified that when autophagy is inhibited either pharmacologically or genetically cells should be classified as undergoing autophagic death (Liu & Levine 2015).

Necrosis or Type III cell death, resulting from depletion of energy resources and/or the direct insult to the cell, is characterised by cellular swelling, followed by permeabilization and rupture of the plasma membrane (Elmore 2007; Golstein & Kroemer 2007).

2.5.2 Cross talk between autophagy and apoptosis

Autophagy can promote either cell survival or death and can protect the cell from various apoptotic stimuli. Although generally a survival mechanism, autophagy is considered to be type II programmed cell death due to the presence of vacuoles in the dying cells and this process is independent of caspase. Moreover, cells in which apoptosis is defective undergo autophagic cell death (Maiuri et al. 2007).

Molecular mechanism(s) of cross talk: The mechanisms that interconnect autophagy and apoptosis involves mitochondrial stability and the energy status of the cell. Sequestration of damaged mitochondria during autophagy prevents the release of cytochrome C and, thereby, apoptosis. Cellular energy levels decide the extent of autophagic activity at any given point in time, and cells adapt to survive and delay apoptosis if they can still generate energy (Rikiishi 2012).

In addition to these processes, common signalling pathways involving PI3/Akt/mTOR and proteins such as, *e.g.*, Beclin1, Atg5, p62, Bcl2 and p53 regulate both apoptosis and autophagy (Saiki et al. 2011). Various proteins regulated in connection with autophagy can interact with the apoptotic machinery, for example, the anti-apoptotic protein Bcl-2 prevents Beclin1 from inducing autophagy by removing it from the class III PI3K complex. Bcl2-Beclin1 act as rheostat that decides whether a cell undergoes apoptosis or autophagy (Pattingre et al. 2005).

Another protein associated with autophagy that can also regulate apoptosis is Atg5. Tumour cells which overexpress Atg5 are sensitized to apoptosis. In such cells Atg5 is cleaved by calpains and the truncated form then shifts to the mitochondria to promote cytochrome C release and activates caspases by interacting with Bcl-xL (Yousefi et al. 2006). The caspases that initiate and execute apoptosis also interlink autophagic and apoptotic pathways (Han et al. 2014; Cho et al. 2009; Zhu et al. 2010).

p53, a tumour suppressor protein which activates both intrinsic and extrinsic apoptotic pathways, activates autophagy by activating the DNA damage-regulated autophagy modulator (DRAM) (Crighton et al. 2006), whereas, cytoplasmic p53 downregulates AMPK and inhibits autophagy (Tasdemir et al. 2008).

These are only a few examples of the crosstalk between these two critical pathways. When the cell is poised to undergo apoptosis, autophagy is switched off. Such multifaceted crosstalk between autophagy and apoptosis is influenced by the cell type, the stage of cell cycle, various stimuli and nutrients (Maiuri et al. 2007; Fan & Zong 2013).

2.6 AUTOPHAGY AND mTOR IN THE GROWTH PLATE

2.6.1 Autophagy in the growth plate

Chondrocytes in the avascular growth plate reside under hypoxic conditions (Fig 8). (Cornelia E et al. 2006), trigger autophagy (Bohensky, et al. 2007).

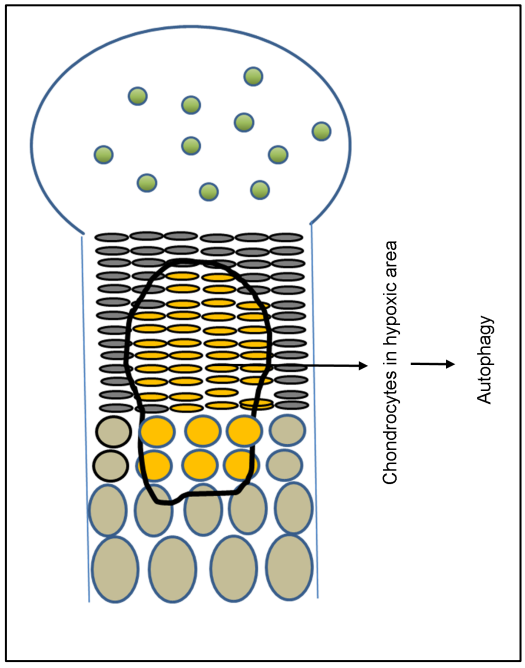

Figure 8. Illustration of hypothetical regions of hypoxia in the growth plate Proliferative chondrocytes in the centre of the growth plate and early hypertrophic chondrocytes reside in hypoxic regions.

Autophagy occurs constitutively in the growth plate (Khatri & Schipani 2008), as reflected in the presence of autophagosomes and LC3 in pre-hypertrophic and hypertrophic zones (Wang et al., 2015), and accumulation of p62 in terminal hypertrophic chondrocytes (Vuppalapati et al. 2015). Moreover, the level of LC3 II in chondrocytes is lowered by culturing in media deprived of glucose and serum and rises simultaneously with the reduction in energy production caused by Baf (Settembre et al. 2008). Autophagy is also an intermediate stage that prolongs the life of hypertrophic chondrocytes (Srinivas & Shapiro, 2006; Bohensky et al. 2007). Various factors such as transcription factor HIF-1α, the energy sensor AMPK, UPR and ER stress are involved in regulating autophagy in chondrocytes (Fig 9).

2.6.2 Regulators of autophagy in the growth plate

Regulation by Hypoxia: To survive in their hypxoic environment, chondrocytes express HIF-1α, a potent stimulator of autophagy, whose loss reduces Beclin1 levels, inhibits autophagy and sensitises these cells to apoptosis (Bohensky et al. 2007).

PIM, a serine/threonine kinase expressed by growth plate chondrocytes, regulates both apoptosis and autophagy by promoting expression of the autophagy proteins Beclin1 and LC3 and inhibiting the apoptotic BAD protein (Bohensky et al. 2007). Moreover, the hypoxic state of the chondrocytes upregulates the uncoupling protein 3 (UCP3) which regulates the mitochondrial membrane potential, through HIF-1α, thereby inducing autophagy (Watanabe et al. 2008).

AMPK regulation of autophagy in the growth plate: AMPK signalling can activate autophagy by inhibiting TOR (Meijer & Codogno 2007), a mechanism that varies with the levels of oxygen and HIF-1α in a cell. AMPK activity in chondrocytes requires HIF-1α and indeed, in the absence of HIF-1α AMPK can inhibit autophagy in chondrocytes under nutrient-deprived conditions (Bohensky et al. 2010).

Factors that regulate the ECM and autophagy in the growth plate: Impairment of autophagy plays a role in the development of chondrodysplasias. Mutations in the genes whose products regulate the ECM, such as CCN2, Sumf1 and FGF3, result in the production of misfolded proteins, which accumulate in the ER, leading to enlargement and stress of this organelle (Nishida et al. 2007). ER stress, in turn, upregulates the Unfolded Protein Response (UPR) in chondrocytes (Watanabe et al. 2008), a mechanism designed to protect cell viability by preventing further protein synthesis while clearing the misfolded proteins with the help of chaperones (Tsai & Weissman 2010). Since autophagy can reduce ER stress by removing misfolded proteins (Ogata et al. 2006), ECM related mutations that cause growth disorders can affect autophagy indirectly (Fig 9).

The connective tissue growth factor (CCN2), a matrix protein that promotes cell-matrix interactions and is essential for linear bone growth (Gao & Brigstock 2006), promotes chondrocyte viability by promoting translocation of NFκB into the nucleus and activating autophagy-related genes (Hall-Glenn et al. 2013).

Sulfatase Modifying Factor 1 (Sumf1) sulphates GAGs and is thus required for effective proteoglycan degradation. Loss of this enzyme leads to the accumulation of GAGs in lysosomes, thereby disrupting lysosomal function and autophagy. Indirectly, this disturbance in autophagy affects chondrocyte viability and enhances FGF signalling, stunting growth (Settembre et al. 2008).

Furthermore, Fibroblast growth factor 3 (FGF3), a well-known negative regulator of endochondral ossification, inhibits all aspects of chondrocyte mechanics. Thus, mutations in this protein cause skeletal abnormalities such as achondroplasia (Bonaventure et al. 1996). FGF3 is co-expressed and interacts with the Atg5-Atg12 complex in chondrocytes, binding to Atg5 and inhibiting its association with Atg12, thereby preventing autophagy (Wang et al. 2015).

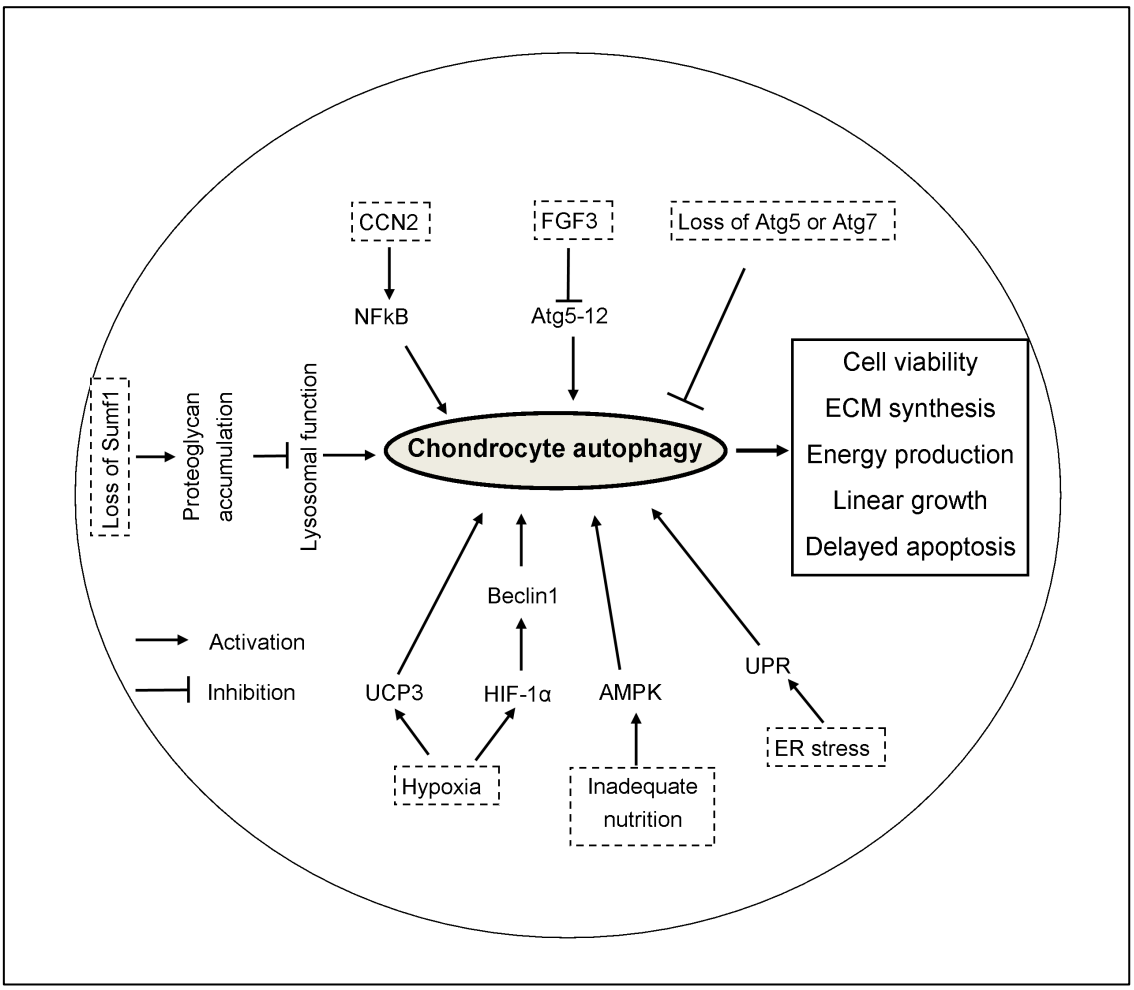

Figure 9. Factors influencing chondrocyte autophagy. Hypoxia, nutrient depletion and ER stress in chondrocytes activates autophagy whereas loss of autophagy genes in chondrocytes and proteoglycan accumation due to loss of sumf1 and defects in CCN2 inhibits autophagy in chondrocytes.

Influence of pharmacological inhibitors on autophagy in linear bone growth: 3MA and CQ both reduce the growth of mouse metatarsal bones (Vuppalapati et al. 2015; Wang et al. 2015). In addition, we have demonstrated that small doses of CQ and Baf promote linear bone growth by enhancing chondrocyte differentiation, an effect dependent primarily on the dose and duration of exposure to CQ and Baf (Newton et al. 2015; Vuppalapati et al. 2015).

Genetic studies concerning the role of autophagy in connection with linear bone growth: The importance of autophagy in chondrocytes was demonstrated directly for the first time by our research group by showing that mice without the Atg5 or Atg7 gene exhibit mild growth retardation due to the reduced proliferation of chondrocytes and their enhanced death mediated by caspases (Vuppalapati et al. 2015). Similarly, loss of Atg7 from mesenchymal cells also reduces bone lengths. Moreover, Atg7 regulates production of extracellular matrix, mainly collagen II (Cinque et al. 2015).

2.7 mTOR IN THE GROWTH PLATE

mTOR directs bone growth by stimulating chondrocyte proliferation and differentiation, as well as, matrix production through a variety of mechanisms.

Pharmacological inhibition of mTOR in the growth plate: Local administration of rapamycin into the tibial growth plate of rabbits reduces bone length by attenuating chondrocyte proliferation and differentiation (Phornphutkul et al. 2009) and similar effects are observed when rapamycin is administered systemically to rats. Rapamycin initially decreases the levels of PTHrP, IGF-1, IGF-BP3, Col II and Col X, while elevating that of Ihh (Sanchez & He 2009). Moreover, mTOR is involved in cell differentiation (Ma et al. 2010, Preitschopf et al. 2014, Araki et al. 2009), as indicated by inhibition of the differentiation of chondrocytes by rapamycin (Sanchez & He, 2009).

Genetic studies supporting the role of mTOR in the growth plate: Conditional deletion of mTOR or its components in chondrocytes provided direct evidence for its role in chondrocyte biology. For instance, deletion of either mTOR or raptor in pre-chondrogenic cells delays apoptosis and decreases cell size, chondrocyte differentiation, matrix production and limb length, with accompanying defects in ossification (Chen & Long 2014). These effects of mTOR and raptor were similar in magnitude, whereas rictor deletion evoked more moderate changes (Chen et al. 2015). Moreover, mTOR is involved in ribosome biogenesis and protein synthesis, chondrocytes without functional raptor contain less protein, suggesting that defective protein synthesis might impair chondrocyte differentiation (Jiang et al., 2016, Chen & Long, 2014).

2.8 AUTOPHAGY AND mTOR IN OSTEOARTHRITIS

2.8.1 Autophagy in healthy and ageing articular cartilage

Since, as described above articular cartilage is avascular, the chondrocytes there have access to only low levels of oxygen and nutrients. Nutrients diffuses from the synovial fluid into the articular cartilage and chondrocytes generate energy primarily through anaerobic metabolism. The microenvironment of chondrocytes activates autophagy constitutively and the autophagy proteins ULK1, Beclin1 and LC3 are all expressed in articular cartilage (Caramés et al. 2010).

Moreover, the hypoxic environment of chondrocytes activates transcription factor HIF-1α, which promotes chondrocyte survival, and HIF-2α, which is expressed at higher levels in osteoarthritic cartilage. Furthermore HIF-2α enhances the expression of FAS, ADAMTS4 and MMPs and promotes cell death and chondrocyte differentiation, thereby attenuating autophagy and contributing to the development of osteoarthritis. Silencing of HIF-2α in chondrocytes induces autophagy, elevates lysosomal activity and lowers mTOR expression (Bohensky et al. 2009).

In both animal and human cartilage affected by OA, the expression of autophagy proteins declines with age, in association with enhanced chondrocyte death (Almonte-Becerril et al. 2010).

2.8.2 Autophagy in connection with osteoarthritis

Defective autophagy in cartilage is associated with the development of osteoarthritis. Early stages of this disease involve elevated autophagy, which helps cells to survive; whereas, in late stages, catabolic signals dominate, leading to chondrocyte apoptosis (Almonte-Becerril et al. 2010). Cells in the superficial and middle zones show features of both autophagy and apoptosis a condition referred to as "chondroptosis" (Roach et al. 2004), whereas cells in the deep zone display only apoptotic features (Almonte-Becerril et al. 2010).

The ATG genes ATG3, ATG5, ATG12, ULK1, LC3 and BECLIN1 are all downregulated in connection with OA. In contrast to the features mentioned above, chondrocytes in the deep layers form clusters that express LC3 (Caramés et al., 2010). In addition to the reduction in autophagy associated with OA, the level of BNIP3, a regulator of both autophagy and apoptosis, is also lowered. Thus, both, a reduction in autophagy and the presence of pro-apoptotic factors in OA cartilage promote apoptosis. Furthermore, mitochondrial dysfunction in chondrocytes elevates ROS production, cell death and mTOR activity and attenuates autophagy (de Figueroa et al. 2015).

Ageing, OA and autophagy: A correlation between reduced autophagic activity and ageing - induced arthritis has recently been reported. Proteomic analysis of Atg5-silenced primary human chondrocytes revealed an elevated level of prelamin, a marker of ageing, and less of the cytoskeletal protein filamin A, along with reduced levels of autophagy proteins (Carames et al. 2015).

ROS, OA and autophagy: Tissue levels of ROS increase with age enhancing the production of MMPs that degrade cartilage. ROS levels are regulated transcriptionally by Forkhead Box O (FOXO), which activates autophagy. FOXO1 and FOXO3 are expressed in healthy cartilage and their expression in the superficial zone declines with age (Akasaki et al. 2014).

Mechanical injury, OA and autophagy: In OA that develops secondary to mechanical injury, the superficial zone is most affected. To adapt to stress-induced injury, chondrocytes in the intermediate and deep zones upregulate autophagy. The protective mechanism cannot guard the chondrocytes effectively against injury, and is later downregulated. Furthermore, rapamycin protects stress-injured chondrocytes from death by inhibiting TNF-α and prevents GAG loss by inhibiting IL-1α, in addition to triggering autophagy (Caramés et al., 2012).

Pharmacological modulators of autophagy in connection with OA

Rapamycin: Systemic (Caramés, Hasegawa, et al. 2012) or local administration (Takayama et al. 2014) of rapamycin in animal models of experimental arthritis reverse the pathological features by inhibiting mTOR and activating autophagy.

Glucocorticoids (GC) are used to treat osteoarthritis because of their anti-inflammatory action. GCs increase ROS levels in chondrocytes, resulting in increased oxidative stress, which in turn activates autophagy (Shen et al. 2015).

Glucosamine; used for supplementary treatment of arthritis, promotes autophagic activity in chondrocytes of the superficial and upper middle zones by inhibiting the Akt/FOXO/mTOR pathway (Caramés et al. 2013).

Genetic evidence supporting the role of autophagy in protecting against OA

The significance of autophagy in maintaining the homeostasis of articular cartilage is demonstrated by the observation that cartilage-specific deletion of chondrocyte Atg5, enhances caspase-mediated apoptosis in these cells and promotes the age-related development of OA (Bouderlique et al. 2016).

2.8.3 mTOR and autophagy in articular cartilage

Genetic studies concerning the role of mTOR in connection with OA: Expression of mTOR in the articular cartilage of humans with OA and animal models of this disease is elevated. Cartilage-specific deletion of mTOR protects mice from developing arthritis by decreasing chondrocyte apoptosis and secretion of MMP13 as well as by inducing autophagy (Zhang et al. 2015).

Recently Vasheghani and colleagues (2015) have shown that the transcription factor PPARγ negatively regulates mTOR. Loss of PPARγ in chondrocytes enhances matrix degeneration, cell death and the level of mTOR. Mice lacking both PPARγ and mTOR display enhanced autophagy and are rescued from arthritis (Vasheghani et al. 2015).

Preclinical observations indicate that inhibition of mTOR and activation of autophagy have beneficial effects on the pathology of OA. Greater research focus on targeting mTOR and autophagy in cartilage might provide new approaches to treating joint pathologies.

2.9 AUTOPHAGY DURING CALORIC RESTRICTION AND ITS INFLUENCE ON METABOLISM

2.9.1 Caloric restriction and metabolism in obesity

A high-caloric diet and improper lifestyle are potent risk factors for obesity, type II diabetes and cardiovascular diseases (CVD (Stanfel et al. 2009, Newgard et al. 2009). Obesity is a rapidly increasing global problem, affecting more than 600 million people, including 11% of all men and 15% of women ("WHO | Obesity and overweight,"). Several reports have concluded that a reduction in body weight subsequently improves parameters associated with diabetes and CVD (Harvie et al. 2011).

Limiting food intake either daily or for a few days each week is called caloric restriction (CR). This age-old practice by people around the world for different purposes has recently awakened more interest due to its beneficial effects on ageing-related changes and ability to lengthen life span (at least in animals) through various mechanisms. Studies on long-term CR in humans pose a significant problem concerning the recruitment, consistency and commitment of participants. Such as study involving 30% lowered food intake by *Rhesus macaques* improved insulin sensitivity and immunological defences, while attenuating brain atrophy and the incidence of cancer, along with the reduction in total body fat (Colman et al. 2009).

Caloric restriction and metabolic changes: "Comprehensive assessment of the long-term effect of reducing intake of energy" (CALERIE) is a two-year randomised controlled study supported by the National Institutes of Health in the United States and designed to assess the physiological effects of 25% CR on healthy individuals. The results revealed reductions in body weight, fat mass and fat-free mass (Stewart et al. 2013), in the levels of the risk factors associated with cardiovascular diseases, such as systolic blood pressure and blood lipid profile, as well as decreased insulin resistance and protection against type II diabetes (Ravussin et al. 2015). Interestingly, abdominal fat content is not altered, despite a decrease in total body fat (Redman et al. 2007). Serum leptin levels have also been reported to be lowered by CR, while the levels of inflammatory mediators such as TNF-α decrease in response to higher levels of glucocorticoids (Yang et al. 2016).

In addition, CR for six months improved the insulin sensitivity of overweight individuals by enhancing beta-cell function and decreasing fat deposits in the liver and adipose tissue (Larson-Meyer et al. 2006), as well as reducing glucose oxidation and metabolism (Franssila-Kallunki et al. 1992). CR decreases serum IGF-1 levels in animal models (Fontana et al. 2010) but does not affect these levels in humans. Intriguingly, the level of circulating IGF-1 has been shown to depend on the protein, rather than the total caloric intake (Fontana et al. 2008). Moreover, a two-year randomised CR study in humans did report a rise in IGFBP-1 levels (Fontana et al. 2016).

2.9.2 The influence of caloric restriction on autophagy

Investigations involving a period of CR without malnutrition are indicative of a relationship between energy requirement and nutrition-sensing pathways that involve mTOR-induced autophagy and IGF-1 (Cuervo 2008). Cellular nutrients signal through the mTOR pathway, regulate autophagy and affect the lifespan and diseases associated with ageing to a considerable extent. Indeed, mice survived 10-15% longer when fed rapamycin in mid- and late-life, but without any effect on the development of disease or ageing (Harrison et al. 2009).

Cavallini and co-workers were first to demonstrate a decrease in autophagic proteolysis with age employing rat hepatocytes and have described how CR initiated at a young age reverses ageing-associated changes (Cavallini et al. 2001). CR for longer periods upregulates the expression of autophagy genes, specifically those encoding Beclin1 and LC3, in human skeletal muscle (Yang et al. 2016).

2.9.3 Continuous versus intermittent caloric restriction

Intermittent caloric restriction involves 75% less food intake on consecutive days e.g., on alternate days or for two days each week (5:2 diet); while continuous CR involves a 25% restriction. Intermittent CR appears to be more practical and results in the same beneficial effects regarding reduced body weight and improved lifespan. Rodents display similar effects on metabolism and lifespan with both intermittent and continuous CR. Recently; these two kinds of CR gave similar weight loss, reduction in insulin levels and enhanced insulin sensitivity in premenopausal women, but noticeable changes in glycemic control only with intermittent restriction (Harvie et al. 2011).

2.9.4 Calorie restriction and Bone mineral density

Unfortunately, long-term caloric restriction, exerts catabolic effects on bone metabolism. Thus 25% calorie restriction in humans for two years result in sustained loss of bone especially at sites prone to fracture such as the neck of femur. Markers of bone catabolism such as the activities of alkaline phosphatase and tartrate-resistant acid phosphatase, were enhanced by CR. Similar studies on CR, but supplemented with exercise showed weight loss in both groups, but reduced bone mineral density only without exercise. These observations indicate that for all tissues careful monitoring and balancing of anabolic and catabolic processes during prolonged CR is essential (Villareal et al. 2016).

2.9.5 Nutrition and linear bone growth

Malnutrition due to inadequate caloric intake or malabsorption stunts linear growth in children, but they usually catch-up once sufficient nutrition is provided (Boersma et al. 2002). Even for a short period of CR reduced the height of the growth plate and numbers of cells in the resting zone of rodent models. Moreover, CR downregulated several genes and

transcription factors essential for linear growth, including HIF-1α. Nevertheless, refeeding resulted in catch-up growth and restoration of growth plate height (Even-Zohar et al. 2008).

In summary, intermittent CR attenuates risk factors for diabetes and CVD. CR in combination with exercise is beneficial for bone homoeostasis, but CR should not be practiced during early growth periods in order to avoid malnutrition and its consequences.

3 PROJECT AIMS

The general aim of this thesis work was to understand the role played by autophagy in connection with chondrogenesis, with a focus on linear bone growth and osteoarthritis. I also wanted to assess the role of autophagy during intermittent caloric restriction.

More specifically, my experiments were designed

- to reveal chondrocyte-specific mechanisms regulating autophagy and mTORC1.

- to explore the role of autophagy in chondrocyte survival and differentiation and its consequent impact on linear bone growth.

- to characterize the significance of autophagy during the ageing of cartilage, as well as its role in the development and progression of osteoarthritis.

- to investigate the regulation of autophagy during intermittent caloric restriction.

4 METHODOLOGICAL CONSIDERATIONS

The following models and methods were used to understand and prove our hypothesis. Detailed explanation about these methods was discussed in constitutive papers.

4.1 MODEL SYSTEMS

Animal models:

Atg5 and Atg7cKO mouse strains: To understand the role of autophagy in linear bone growth and osteoarthritis, mice with deficient autophagy genes Atg5 or Atg7 in chondrocytes were generated using cre–lox approach. Tissue specific genetic modification is a powerful tool to study the effect of gene expression and function in detail and provides better insight in understanding the gene function at tissue level. The main strategy lies in inserting lox p sites around functional part of the gene of interest without disturbing the regular gene function. Cre is introduced into mouse embryonic stem cell genome driven by cell specific promoter. Cre mediates the recombination between the two lox P sites when the cells start expressing the promoter.

Transgenic mice with collage type II promoter driven cre (Col2-Cre) were used to ablate the function of gene of interest in all the experiments with animal models. Collagen type II gene is expressed specifically by chondrogenic cell lineage and Col2-Cre allows selective recombination of the floxed gene in tissues expressing collagen II, mainly chondrocytes.

Atg5cKO mice: Exon 3 of the Atg5 gene in the mouse genome was flanked by lox p sites (Atg5fl/fl). These mice were described in detail by Hara T et al (Hara et al. 2006). To generate Atg5 fl/fl, Atg5 fl/+ and Atg5 -/- (Atg5cKO) mice, Col2-Cre mice were crossed with Atg5fl/fl mice.

Atg7cKO mice: Exon 14 of Atg7 gene was flanked by lox p sites (Atg7fl/fl). These mice were described in detail by Komatsu M et al (Komatsu et al. 2005). Atg7fl/fl, Atg7 fl/+ and Atg7-/-(Atg7cKO) mice were generated by crossing Atg7fl/fl mice Col2-Cre mice.

mT/mG double fluorescent reporter cre mice: To study the effectiveness and extent of cre recombination Atg5fl/fl mice were crossed with this double fluorescent reporter mouse. Membrane targeted tomato was expressed before cre mediated recombination and green fluorescent protein was expressed after effective cre recombination (Muzumdar et al. 2007).

Mice were sacrificed on postnatal day 4, one, two, six and twelve months and genotyping was done using DNA from tail biopsy. Successful cre recombination and ablation of gene was confirmed by PCR and protein levels of Atg5, or Atg7 and markers of autophagy LC3 I, LC3II and p62 were visualized by western immunoblot.

Mouse metatarsal culture: Mouse metatarsal cultures from wild type and autophagy deficient mice were used to study the direct effects of autophagosome inhibitors and to monitor their effect on growth in real time. The advantage of metatarsal organ cultures is that the chondrocytes reside within the growth plate and maintain cell to cell and cell to matrix interaction and permits to analyze chondrocyte proliferation, viability, differentiation and matrix production. The main disadvantage of this model is lack of blood supply, mechanical

force and systemic factors. The absence of systemic influences provides an additional advantage when studying the direct effect of drugs on bone growth.

Detailed description of the culture conditions is found in paper I and II. Briefly, metatarsal bones were cultured for 6 days in serum free medium. Images of the bones were captured and measured on day 0, 2, 4 and 6 with image J and medium was changed every 2 days. Day 0 is the day where the bones were dissected and placed in the culture. On day six metatarsal bones were fixed in formaldehyde and decalcified with EDTA before advancing to tissue processing and sectioning.

Human growth plate cultures: Human growth plate cultures were used to provide proof of concept to our experiments with rodents and interpretations from these cultures provides an insight in understanding the mechanisms in more physiological settings that can be linked to *in vivo* findings. Human growth plate tissue from proximal tibia and distal femur was obtained during epiphysiodesis surgery (to cease further bone growth in children with tall stature). The human growth plate biopsies were collected using bone marrow biopsy needle and transferred to tubes containing DMEM/F12 media supplemented with gentamycin (50µg/ml) on ice. The biopsy was then cut into thin slices and each slice was cultured in separate well in a 24 well plate for 3 days. Autophagy inhibitors were added to the culture media on day 0. The culture media and concentration of drugs were same as in mouse metatarsal culture. On day 3, the tissue was fixed in formaldehyde for 24 hours, decalcified with EDTA for a week at 4ºC before tissue processing and sectioning.

Cell cultures: RCJ3.C5.18 rat mesenchymal chondrogenic cell line (C5.18): To validate our *in vivo* and mouse metatarsal organ culture data and to dissect the molecular mechanism of autophagy and mTOR and to understand the mechanism of cell death upon inhibition of autophagy process we used RCJ3.C5.18 nontransformed mesenchymal rat chondrogenic cell-line (C5.18). These cell line has the capability to undergo chondrogenic differentiation and acquire chondrocyte phenotype and histochemical markers like that of chondrocytes of growth plate. Briefly after reaching confluence cells were differentiated in presence of ascorbic acid and ß-glycerophosphate and treated with autophagy inhibitors after terminal differentiation.

4.2 ANALYSIS OF BONE GROWTH, GROWTH PLATE AND ARTICULAR CARTILAGE STRUCTURE

Quantitative histology of growth plate and articular cartilage: Histomorphometric analysis was conducted using hematoxylin and eosin stained sections. The size of the hypertrophic chondrocytes was calculated by taking an average of 25 measurements in the middle of the hypertrophic zone of the distal growth plate of metatarsal bone using Image J. Morphometric analysis of the growth plate – Growth plate height, proliferative and hypertrophic zone height, terminal hypertrohic chondrocyte cell size (cells with intact lacuna close to chondro-osseous junction) was analyzed by examining proximal tibial growth plate.

Articular cartilage morphology and osteoarthritis scoring was performed on safranin O stained sections of medial joint.

Peripheral Quantitative Computerised Tomography (pQCT): pQCT is useful tool to measure bone mineral (BMD) density and true volumetric density of trabecular and cortical bone separately. The advantage of pQCT is that it measures bone strength (Stress strain index).

In our study tibia from control and Atg5cKO mice at 2 months of age were analyzed. Metaphyseal region of tibia was used to measure trabecular BMD and diaphyseal region to measure cortical BMD, area and thickness.

4.3 HISTOLOGICAL STAINING

Von Kossa staining: Von Kossa staining is used to detect mineralization by identifying calcium deposits. Photochemical reaction with development of black colour occurs by replacing calcium with silver at mineralization area. Non-decalcified metatarsal bones were treated with silver nitrate solution under light to detect mineralization.

Safranin O/Fast green staining: It is used to detect cartilage. Tissue sections were first stained with Weigerts Hematoxylin followed by immersion in acid ethanol and later washed in distilled water. Sections were then stained with fast green, 1% acetic acid and finally with safranin O solution.

Tartrate Resistant Acid Phosphatase (TRAP) or ACP5 Staining: Presence of TRAP is hallmark of osteoclasts. Sections from metatarsal bones treated with either control or Baf were used for this purpose. Briefly, slides were incubated in wash-buffer (0.92% sodium acetate solution, 1.14 % L-tartaric acid, 0.28% acetic acid, pH 5.0) containing 0.02% naphthol AS-BI phosphate. Slides were then placed in pre-warmed (37°C) wash buffer containing 0.01% sodium nitrite and 0.01% parasonaline chloride, and incubated at room temperature.

4.4 DETECTION OF CELL PROLIFERATION

5-bromo-2–deoxyuridine (BrdU) labeling: Identification and quantification of proliferating cells is important in understanding the cellular responses to various external and internal stimuli either by drugs, diseases or genetic alterations etc.

BrdU is a synthetic nucleoside (modified uridine) analogue to thymidine. The basic principle of this method is, replicating cells with newly synthesized DNA during S phase of cell cycle incorporate BrdU in place of thymidine.

To evaluate chondrocyte viability and proliferation in *in vivo* mice with and without autophagy in chondrocytes were injected with BrdU intraperitoneally. BrdU was injected 2 hours before sacrifice to postnatal day 4, mice and older mice received two doses of BrdU 24 and 4 hours prior to sacrifice. For *in vitro* labelling, BrdU was added to the existing culture media to label proliferating cells 2.5 hours before terminating the experiment.

The labelled cells in the tissue sections from both *in vitro* and *in vivo* experiments were detected using monoclonal anti BrdU antibody.

2-Ethynyl-2-deoxyuridine (EDU) labelling: Proliferating cells with newly synthesized DNA were labelled with 5-ethynyl-2′-deoxyuridine (EdU) and after labelling with EdU the cells were fixed, permeabilized and labelled with azide dye in presence of copper sulphate and

ascorbic acid. Detection is based on a chemical reaction between azide and ethynyl groups catalyzed by copper.

4.5 DETECTION OF APOPTOSIS

TUNEL assay: The terminal deoxynucleotidyl transferase-(tdt)-mediated deoxy UTP nick-end labeling (TUNEL) assay was used to detect apoptotic cells. The classical feature of apoptosis is DNA fragmentation with double stranded breaks exposing 3 hydroxyl groups (3'-OH). The basic principle of TUNEL is tdt identifies nicks in the DNA and catalyzes labelling of free 3'-OH groups with dUTP nucleotides. The tissue sections were digested with 20µg/ml of proteinase K and incubated with enzyme and label solutions contained in *In Situ* cell detection kit from Roche and later the nucleus was labelled with DAPI thereby enabling to detect the cells with DNA fragmentation.

Cytochrome C release: To investigate the apoptotic signalling in Baf treated C5.18 cells or metatarsal bones cytochrome c assay was performed. Cytochrome C (cytc) is the enzyme in the respiratory chain located on the outer surface of inner mitochondrial membrane. Release of Cytochrome C into cytosol triggers cascade of apoptotic events by activating caspases. Differentiated C5.18 cells were treated with Baf for 24 hours lysed in RIPA buffer and cell fraction was used for detection of cyt C by western immunoblot.

4.6 DETECTION OF GENE EXPRESSION

Quantitative real-time reverse transcription PCR (RT-PCR): RT-PCR was used to analyze the mRNA levels encoding matrix proteins-collagen I, collagen II, Collagen X, Alkaline phosphatase in C5.18 cells treated with or without autophagy inhibitor Baf. RNA was isolated from differentiated C5.18 cells and metatarsal growth plate heads using trizol method and converted to cDNA. qPCR was carried out using commercially available TaqMan probes.

In situ Hybridization: This technique involves detection of specific sequence of nucleic acid using labelled complementary strand of nucleic acid, called probe. The tissue was permeabilized with proteinase K, acetylated and incubated with RNA probe (100ng of probe) (ColX and Ihh) diluted in hybridization solution overnight followed by rigorous washing and probe detection using digoxinin labelled antibody. Development of the signal using coloured substrate like NBT and BCIP solutions to visualize the probe.

4.7 DETECTION OF PROTEIN EXPRESSION

Immunohistochemistry: This technique was used in our experiments to detect autophagy marker proteins – LC3 and p62, pS6 - readout of mTOR and chondrocyte differentiation marker protein collagen X in the growth pate and articular cartilage sections.

In short, heat induced antigen retrieval of the sections with citrate buffer was performed. The sections were quenched with hydrogen peroxide and incubated with primary and secondary antibodies followed by signal detection by amplifying with avidin biotin system.

Immunocytofluorescence: This method involves detecting proteins in cells. FLCN and LAMP2 colocalization was analyzed with this method. Briefly cells fixed in formalin were

permeabilized with triton and incubated with primary antibody and then with fluorescent labelled secondary antibody. Images were captured with confocal microscope and co-localization was analyzed with image J software.

Western immunoblotting: This method involves detection and quantification of proteins. Autophagy marker proteins p62, LC3 isoforms I and II, mTOR pathway proteins - mTOR, 4E-BP1, p70S6K, pS6, apoptotic proteins - cleaved caspase -3 and -9 and cytochrome C were detected using this method.

Briefly differentiated C5.18 cells and metatarsal growth plate cartilage treated with or without Baf and PBMCs from subjects who are on 5:2 diet was lysed in laemlyis lysis buffer and subjected to SDS-PAGE.

4.8 DETECTION OF LYSOSOMAL FUNCTION

Cathepsin B assay: Cathepsin B is a lysosomal proteinase involved in intracellular degradation and recycling of proteins. The principle of this method is to detect active cathepsin by cell permeabilization using non-toxic reagent provided with the kit. The non-toxic reagent is cleaved in presence of cathepsins to produce fluorescent signal.

4.9 SURGICAL INDUCED MODEL OF OSTEOARTHRITIS

Partial Medial Meniscectomy Model (MMT): Meniscus is crescent shaped fibrocartilage structure that acts as shock absorber between tibia and femur and medial meniscotibial ligament (MMTL), which anchors medial meniscus to tibia. MMT is used to induce osteoarthritis in mice as mice do not develop OA spontaneously. OA developed due to mini-open partial medial meniscectomy resembles OA in humans.

Mice with and without autophagy (Atg5fl/fl and Atg5cKO mice) at 2 months of age were anaesthetized with isoflurane, skin incision was made from distal patella to proximal tibia plateau, joint capsule was opened and fat pad was dissected to expose MMTL. MMTL of right leg was transacted at the level of anterior attachment of medial meniscus to tibial plateau and left leg was used as internal control.

4.10 RANDOMIZED CONTROLLED STUDY OF INTERMITTENT CALORIE RESTRICTION

Diabetic or non-diabetic subjects with obesity were recruited after formal consent. All the participants were placed on 5:2 diet for six months. Basal anthropometric, metabolic parameters were recorded before starting the study and after six and twelve weeks.

5 RESULTS AND DISCUSSION

5.1 THE EFFECTS OF INHIBITING AUTOPHAGY ON LINEAR BONE GROWTH (PAPER I)

To examine these questions, we cultured mouse metatarsal bones *ex vivo* in the presence of the inhibitors of autophagy, bafilomycin (Baf) and chloroquine (CQ). Both stimulated longitudinal bone growth to the same extent as IGF-1, a very well-characterized promoter of bone growth. Increased linear bone growth was associated with larger hypertrophic chondrocytes (Fig 10) and more production of collagen X. Slightly more apoptosis and less proliferation were observed in the chondrocytes, mainly those in the proliferative zone, of Baf treated bones.

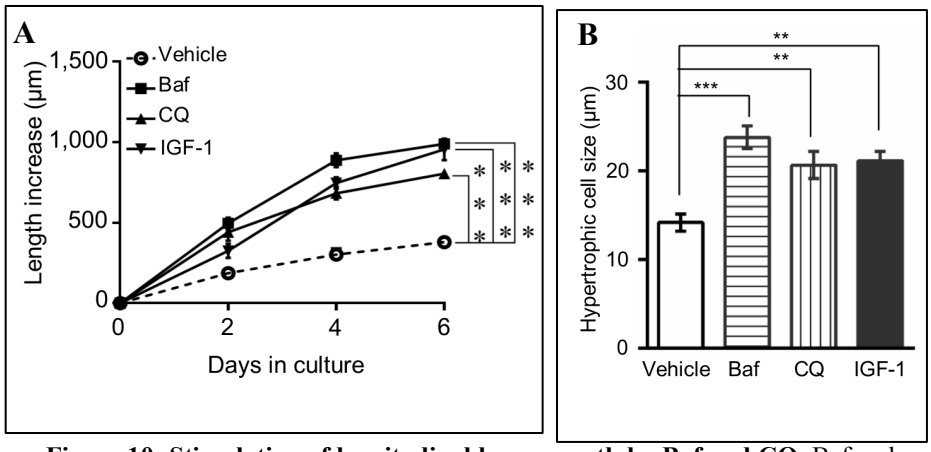

Figure 10: Stimulation of longitudinal bone growth by Baf and CQ. Baf and CQ stimulated longitudinal bone growth of mouse metatarsal bones when cultured *ex vivo* (A) and baf and CQ increased size of terminal hypertrohic chondrocytes (B).

These results, led us to speculate that inhibition of autophagic flux promotes longitudinal bone growth. To test this proposal, we then treated mouse metatarsal bones from Atg5cKO mice with Baf. Interestingly, Baf stimulated the growth of these bones to the same extent as those from control animals indicating that the enhancement of growth caused by Baf is independent of autophagy.

Most longitudinal bone growth (60 -70%) is accounted for by the hypertrophy of chondrocytes (Bush et al. 2010). Cell size is regulated by multiple signalling pathways, among which mTORC1 is fundamental. The growth plate of Baf and CQ treated bones displayed a pronounced increase in phosphorylation of ribosomal S6 protein, a downstream target of the mTORC1 pathway. Moreover, the stimulatory effect of both Baf and CQ on bone growth was abrogated by Torin1, an inhibitor of mTOR.

In confirmation, rapamycin another inhibitor of mTOR reduced longitudinal bone growth and reduced chondrocyte size in rabbits (Phornphutkul et al. 2009). However, the role of mTOR

should be confirmed definitively through genetic studies, since pharmacological modulators may exert many unknown actions.

Our observation that Baf and CQ activate mTORC1 is contrary to the well established principle that lysosomes are required for activation of mTORC1. Translocation of mTORC1 to the lysosomal surface, which depends on the availability of amino acids, is essential for activation of its kinase activity. V-ATPases are blocked by Baf, which should accordingly prevent mTORC1 activation (Sancak et al. 2010). Thus, our findings of elevated mTORC1 upon Baf treatment require further investigation.

The underlying mechanism was dissected by employing the RCJ 3.1C5.18 rat mesenchymal cell line, where, mTORC1 was activated (increased pS6) only in those cells that differentiated into chondrocytes. Since mTORC1 was downregulated in hepatocytes, MEFs and osteoblasts, the mTORC1 activation observed is chondrocyte cell specific.

Since translocation of mTOR to the lysosomal surface requires amino acids, we examined whether nutrition influenced activation of chondrocyte mTOR in the presence of Baf. This effect was independent of nutrition and Baf enhanced phosphorylation of S6 in C5.18 cells cultured in the presence or absence of serum or of essential amino acids. Furthermore, the observation above was confirmed by localization of folliculin, an amino acid-dependent activator of mTORC1, to lysosomes in the presence or absence of Baf.

Bafilomycin acts by inhibiting V-ATPases, which acidify cellular compartments or organelles by pumping in protons. Such acidification is required for the turnover of cytoplasmic constituents (Mauvezin & Neufeld 2015) . The macrolide drug concanamycin acts in the same manner as Baf (Li et al. 2013) and also increased phosphorylation of S6 in C5.18 cells and downregulated in MEFs.

In support of our findings when osteoclasts are treated with CQ or ammonium chloride, activated mTOR accumulates in lysosomes (Hu et al. 2016). Chondrocytes and osteoclasts are terminally differentiated and activation of mTOR might be unique to such cells. Another plausible explanation is degradation of mTOR by lysosomes. Treatment of osteoclasts with CQ or MG132 (proteosomal inhibitors) enhanced the levels of both mTOR and P-mTOR, suggesting that mTOR is degraded in lysosomes and proteasomes (Hu et al. 2016). However, we did not observe any change in the levels of mTOR and P-mTOR upon treatment with Baf.

Another possible explanation is that in chondrocytes the interaction between lysosomal V-ATPases and mTORC1 might differ from that in other cell types, rendering activation of mTORC1 nutrient-independent.

In conclusion, we documented the novel observation that inhibition of V-ATPase by Baf or lysosomal function by CQ stimulates longitudinal bone growth by enhancing chondrocyte hypertrophy. Such inhibition activates mTORC1 signalling to certain extent in a manner specific to chondrocytes.

5.2 CHONDROCYTE-SPECIFIC DELETION OF THE AUTOPHAGY-RELATED ATG5 OR ATG7 GENE LEADS TO MILD GROWTH RETARDATION (PAPER II)

Although, autophagy has been shown to be constitutively active in chondrocytes and autophagic proteins detected in the growth plate (Bohensky et al., 2007, Bohensky et al., 2010), the lack of direct observations *in vivo* limits our understanding of the physiological role of autophagy in connection with linear bone growth. Therefore, we abrogated the autophagy-related ATG5 or ATG7 gene in chondrocytes and characterized growth plate morphology, chondrocyte proliferation, apoptosis and differentiation at 4 days, one month and two months of age, as well as bone length at one, two and six months of age.

Autophagy-deficient Atg5cKO and Atg7cKO mice exhibited retardation of axial and appendicular skeletal growth at two months of age, after which the proportional decrease in limb length remained constant. This impairment was associated with attenuated chondrocyte proliferation and elevated chondrocyte death (Fig 11). We also observed a reduction in bone mass in the Atg5-cKO mice at two months of age, due to the loss of autophagy in osteoblasts and osteocytes as a result of the expression of the collagen II promoter in osteoblast precursor cells.

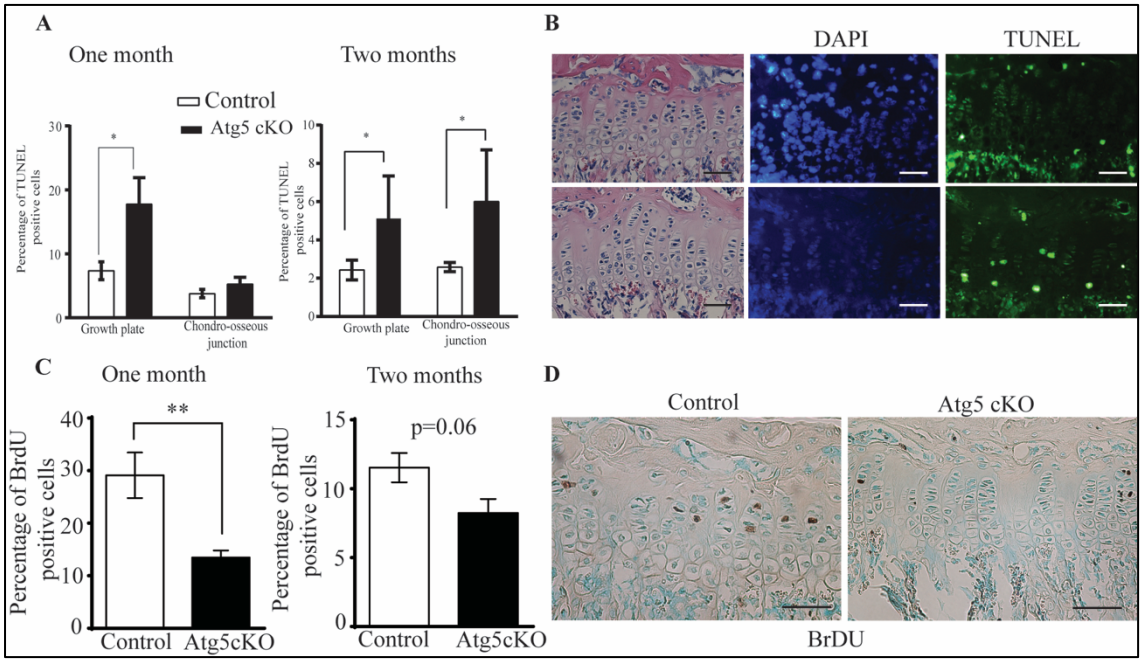

Figure 11: Inhibition of autophagy by ablation of Atg5 increased cell death and decreased chondrocyte proliferation. Percentage of TUNEL cells in the growth plate of control and Atg5cKO mouse (A) and the representative image (B). Percentage of BrdU positive cells in growth plate of control and Atg5cKO mice at one and two months of age (C) and their representative images (D).

In Atg5cKO mice chondrocyte survival was lowered even much earlier than the changes in bone length became obvious. Apoptotic cells were present throughout the growth plate in these mice at one month of age, whereas apoptotic cells were limited mostly to the hypertrophic zone at two months of age in both Atg5- and Atg7-cKO mice. Similarly, chondrocyte proliferation in Atg5cKO mice declined significantly at one month of age

indicating that autophagy is a crucial regulator of cell viability, finally reducing chondrocyte survival and compromising bone length. In line with the general view of autophagy as a mechanism for promoting cell survival, our results show that autophagy is involved in sustaining cell viability under stressful conditions.

Moreover, our conclusions have gained support from the recent observation that col2-cre-mediated loss of Atg7 results in growth retardation, indicating that defective production of collagen II causes the reduction in bone length. In addition, the decrease in bone length was greater than in our case (Cinque et al. 2015). In contrast to their observation on ECM synthesis we observed no change in the level of collagen II mRNA in Atg5cKO metatarsal bones cultured for a short period (unpublished data).

A disparity in the function of autophagy-related genes has been reported in photo-irradiated murine hepatoma cells deficient in Atg5 or Atg7 (Kessel et al. 2012). Thus, we might have overlooked the variations in the function of these genes in connection with skeletal regulation because of our initial similar findings. Irrespective of the changes in chondrocyte death and proliferation, the architecture of the growth plate was not disturbed and no differences in zonal height or the size of terminal hypertrophic chondrocytes were observed. Moreover, there were no differences in proliferation and apoptosis in Atg5cKO mice on postnatal day four. The decrease in bone length was similar in males and females, indicating a lack of any gender influence.

To understand the molecular mechanism(s) by which autophagy retards the growth, we utilized cultures of mouse metatarsal bones and human growth plate, as well as the C5.18 rat mesenchymal chondrogenic cell line. Inhibition of autophagy with higher concentrations of Baf or 3MA abolished the growth of metatarsal bones completely, although lower concentrations promoted growth. Inhibition of growth was associated with enhanced cell death and attenuated proliferation. Our findings with C5.18 cells and human physis tissue were similar, confirming our earlier results *in vivo*. The stimulatory effect of lower levels of Baf on bone growth might reflect enhancement of chondrocyte differentiation (Newton et al. 2015).

In itself, the microenvironment of the growth plate (low oxygen tension, low nutrient supply) activates autophagy constitutively. It has been proposed that hypertrophic chondrocytes utilize autophagy to prolong their lifespan before undergoing apoptosis (Srinivas & Shapiro 2006) and several studies confirm an association between apoptosis and autophagy. Loss of Atg5 or Atg7 triggers apoptosis in hepatocytes (Amir et al. 2013) and suppression of Beclin1 in N1511 chondrocytes is associated with increased levels of the proapoptotic protein BID and caspase 8, as well as decreased cell viability (Bohensky et al. 2007). In consistent with these observations, we detected elevated levels of cleaved caspases 3 and 9 and of PARP, as well as release of cytochrome C into the cytoplasm upon inhibition of autophagy by Baf or 3MA. This indicates that the mechanism underlying cell death is caspase-dependent and involves the intrinsic apoptotic pathway.

Interestingly, we observed that p62, a marker of inhibited autophagy, accumulates in hypertrophic chondrocytes throughout the growth plate of Atg5cKO mice on postnatal day four, but at one month of age, only in the hypertrophic chondrocytes of both control and Atg5cKO mice. Thus, p62 might sensitize chondrocytes to apoptosis, and indeed, p62 was shown to induce both extrinsic (Zhang et al. 2013) and intrinsic apoptosis (Chen et al. 2014). Alternatively, compensatory mechanisms such as increased proteosomal degradation may be involved in clearing p62. An Atg5/Atg7-independent pathway has been reported (Nishida et al. 2009) and chaperone-mediated autophagy might compensate for the loss of Atg5 or Atg7 in chondrocytes.

In conclusion, we provide the first direct evidence for the role of autophagy in linear bone growth. Autophagy promotes cell viability and inhibition of this process attenuates linear bone growth in mice, as a result of activation of caspase-mediated chondrocyte cell death.

5.3 INHIBITION OF AUTOPHAGY IN CHONDROCYTES BY DELETION OF ATG5 PROMOTES AGE-RELATED OSTEOARTHRITIS IN MICE (PAPER III)

Numerous reports have shown that autophagy-related genes are down-regulated with advancing age as well as in osteoarthritic cartilage (Jallali et al. 2005). Since, chondrocytes are the only cells resident in articular cartilage and express principally collagen II, we decided to explore the development of osteoarthritis in autophagy-deficient mice.

Here, we demonstrated for the first time that deletion of Atg5 in chondrocytes was associated with the development of age-related OA, resulting from enhanced chondrocyte death in articular cartilage. This death of chondrocytes was observed at 4 months of age, whereas the histological changes associated with age-related OA were observed at 6 months of age (Fig 12).

The superficial zone of the articular cartilage in Atg5cKO mice exhibited the first histological signs of OA, including fibrillations and a reduction in the level of proteoglycans. The destruction of cartilage continued and by the age of one year the male Atg5cKO mice developed obvious OA, with 29% of these animals displaying complete destruction of articular cartilage at this time point. Female Atg5cKO mice were more resistant, showing the earliest signs of cartilage degradation only at one year of age.

Protection against the development of OA by oestrogen is well documented (Roman-Blas et al. 2009) and the novel finding here was that the mice were protected even in the absence of a functional Atg5 gene, suggesting gender differences in autophagy. Several studies have reported sex differences in autophagic activity in connection with conditions such as cancer and neuronal starvation and, moreover such differences in constitutive autophagy were also observed in rats (Oliván et al. 2014). In contrast, we detected no obvious sex differences with respect to the role of autophagy in connection with linear bone growth in our earlier study, suggesting that such sexual dimorphism might depend on the type of tissue and its physiological and/or pathological status. In addition, analysis of histological sections of

articular cartilage revealed a higher Osteoarthritis Research Society International (OARSI) score for Atg5cKO males than females, with no such sex differences in the control animals.

Both autophagy proteins such as Atg5, Atg12, LC3 and ULK1 and anti-apoptotic proteins are downregulated in human OA (Lotz & Carames 2011) (Musumeci et al. 2015). To elucidate the mechanism involved in cell death, we examined cleavage of caspases-3 and -9 in the articular cartilage of Atg5cKO mice and found significant increases (Fig 12). In a similar manner, our previous observations on C5.18 cells indicated that blocking autophagy with Baf resulted in the release of cytochrome C from mitochondria, there by activating the intrinsic apoptotic pathway leading to chondrocyte death (Vuppalapati et al. 2015). Therefore, we speculate that a similar mechanism occurs in the articular cartilage of Atg5cKO mice. The characteristic feature of OA is matrix degeneration by matrix metalloproteinases primarily MMP13, and the activity of MMP13 in the cartilage of Atg5cKO mice was elevated.

We also found that a partial medial meniscal tear accelerated the development of OA in both control and Atg5cKO mice to a similar degree, more so in male than female animals. The entire destruction of cartilage in these model mice might have influenced the effect of autophagy. Furthermore, the effect of regulators of autophagy such as mTOR should be considered, since significant upregulation of mTOR has been observed in OA cartilage from both humans and experimental animals.

Loss of mTOR from articular cartilage is beneficial, resulting in the up-regulation of autophagy-related genes such as ULK1, Atg5 and LC3. The mice were even protected from post-surgical OA (Zhang et al. 2015, Pal et al. 2015). Rapamycin, an mTOR inhibitor also protected against cartilage degeneration by up-regulating autophagy-related genes in a model of trauma-induced OA (Takayama et al. 2014). mTOR has multidimensional functions in addition to regulating autophagy and its effect on cartilage homeostasis confirms our findings of the protective role of autophagy against OA.

Another interesting finding here was the attenuated accumulation of p62 in articular cartilage with advancing age. p62 aggregated throughout the articular cartilage of Atg5cKO mice at two months of age and the extent decreased thereafter, remaining, however, higher than in control animals. We have observed similar distribution of p62 in growth plate cartilage previously (Vuppalapati et al. 2015), as well as, no changes in cell death in the cellularity of articular cartilage at 6 months of age. Such changes might result from some compensatory or Atg5-independent pathway for autophagy.

In conclusion, this investigation shows that autophagy plays an important role in maintaining the homeostasis of articular cartilage and loss of this process by chondrocytes leads to cartilage degeneration and the development of age-related osteoarthritis, especially in male mice in association with reduced cell viability and caspase-mediated cell death.

5.4 INTERMITTENT CALORIC RESTRICTION REDUCES BODY WEIGHT AND FAT IMPROVES METABOLIC PARAMETERS AND ACTIVATES AUTOPHAGY (PAPER IV)

Obese individuals with and without diabetes were subjected to intermittent caloric restriction (a 5:2 diet) for 12 weeks. Anthropometric and metabolic parameters were measured before starting, as well as six and twelve weeks later. This intervention significantly reduced body weight, the body mass index and % of fat in both diabetic and non-diabetic subjects.

Among the very few studies involving long-term caloric restriction in humans, weight loss was detected after as little as three weeks of intermittent CR that differed from ours (Heilbronn et al. 2005). Moreover, the reduction in body weight has been reported to be more greater with alternate day than intermittent fasting (Barnosky et al. 2014), and the % decrease in fat is more pronounced with intermittent than continuous CR (Varady 2011). Moreover, alternate-two day CR lowers serum levels of inflammatory mediators (Johnson et al. 2006), suggesting that intermittent CR may be a better strategy for reducing body weight.

Both our own and previous observations show that risk factors associated with cardiovascular disease, such as blood pressure and lipid profile, can be improved by CR (Eshghinia & Mohammadzadeh 2013). Furthermore, the increase in insulin sensitivity resulting from CR with time is proportionate to the weight loss (Klempel et al. 2012, Harvie et al. 2011). Although exercise helps reduce weight during CR, it did not augment the beneficial effects on insulin sensitivity (Fayh et al. 2013).

Starvation is the physiological stimulus for autophagy and CR has indeed been reported to ameliorated ageing-related changes by activating autophagy (Morselli et al. n.d.). So, except for a tendency towards an increase in the LC3 II/LC3 I ratio, we observed no change in the expression of autophagy proteins following six weeks of CR. One of our diabetic subjects with elevated levels of IGFBP-1, indicative of enhanced insulin sensitivity and reduced IGF-1 levels, exhibited higher autophagic activity.

Fontana and colleagues proposed that circulating levels of IGF-1 during CR are reduced by lowering protein intake (Fontana et al. 2008). Moreover, it has been reported recently that two-year of CR in a non-obese population significantly reduced their circulating levels of IGF-1, while increasing IGFBP-1 levels (Fontana et al. 2016). IGF-1 acts through the Akt/mTOR pathway to regulate autophagy negatively (Bains et al. 2009). Accordingly, CR might influence the level of IGFBP-1 concentration by reducing the level of IGF-1 by autophagy.

In conclusion, CR reduces body weight and fat mass while improving risk factors associated with cardiovascular diseases in both diabetic and non-diabetic obese individuals, by activating autophagy moderately, mainly in diabetic subjects.

6 CONCLUSIONS

1. Inhibition of the autophagy by lysosomal blockers bafilomycin A1 and chloroquine promotes the linear growth of metatarsal bones. This stimulation is due to enhanced chondrocyte differentiation and is independent of autophagy. Inhibition of lysosomal V-ATPases by Baf and of lysosomal activity by CQ activates mTORC1 in chondrocytes, by a mechanism that is chondrocyte-specific and independent of both autophagy and nutritional status.

2. Autophagy is involved in chondrocyte survival and inhibition of this process by deleting the autophagy-related Atg5 or Atg7 gene resulted in mild growth retardation. This reduction in bone length reflects both attenuated cell proliferation and enhanced cell death. Moreover, inhibition of autophagy promotes apoptotic cell death through the cleavage of caspases-3 and -9 and release of cytochrome C from mitochondria.

3. Lack of autophagy in chondrocytes in the articular cartilage promotes age-associated degenerative changes, leading to the development of osteoarthritis. These changes are associated with elevated cell death mediated by caspases, especially -3 and -9.

4. Intermittent caloric restriction according to a 5:2 diet improves anthropometric and metabolic factors in an obese population with or without type II diabetes. Furthermore, caloric restriction activates autophagy in diabetic patients and this stimulation is more pronounced in those whose insulin sensitivity is improved by caloric restriction.

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